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Forum Review

Role of Redox Reactions in the Vascular Phenotype of Hyperhomocysteinemic Animals

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ABSTRACT

Hyperhomocysteinemia is a risk factor for cardiovascular disease, stroke, and thrombosis. Several animal models of hyperhomocysteinemia have been developed by using both dietary and genetic approaches. These animal models have provided considerable insight into the mechanisms underlying the adverse vascular effects of hyperhomocysteinemia. Accumulating evidence suggests a significant role of altered cellular redox reactions in the vascular phenotype of hyperhomocysteinemia. Redox effects of hyperhomocysteinemia are particularly important in mediating the adverse effects of hyperhomocysteinemia on the endothelium, leading to loss of endothelium-derived nitric oxide and vasomotor dysfunction. Redox reactions also may be key factors in the development of vascular hypertrophy, thrombosis, and atherosclerosis in hyperhomocysteinemic animals. In this review, we summarize the metabolic relations between homocysteine and the cellular redox state, the vascular phenotypes that have been observed in hyperhomocysteinemic animals, the evidence for altered redox reactions in vascular tissue, and the specific redox reactions that may mediate the vascular effects of hyperhomocysteinemia. *Antioxid. Redox Signal.* 9, 1899–1909.

INTRODUCTION

Hyperhomocysteinemia is an established risk factor for cardiovascular disease, stroke, dementia, and vascular thrombosis (31). Many of the clinical manifestations of hyperhomocysteinemia mimic those seen in other common vascular disorders such as diabetes mellitus, hypercholesterolemia, and hypertension (10, 30, 51). In the last decade, several novel animal models of hyperhomocysteinemia have been developed. Insights gained from these animal models have established that experimental hyperhomocysteinemia produces endothelial dysfunction and vascular hypertrophy and accelerates the development of arterial thrombosis and atherosclerosis (45). Emerging evidence suggests that an altered cellular redox state may play an important role in mediating these adverse vascular effects of hyperhomocysteinemia. Here, we review the vascular phenotypes observed in hyperhomocysteinemic animals and assess the evidence that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are key mediators of hyperhomocysteinemic vascular disease.

HOMOCYSTEINE METABOLISM AND THE CELLULAR REDOX STATE

Hyperhomocysteinemia, defined as elevation of total homocysteine (tHcy) in blood plasma (55), results from metabolic abnormalities in homocysteine remethylation or transsulfuration (Fig. 1). In the homocysteine-remethylation pathway, a methyl group is transferred to homocysteine *via* one of two distinct enzymes: (a) methionine synthase (MS), which uses 5-methyltetrahydrofolate as a methyl donor and requires vitamin B_{12} as a cofactor, or (b) betaine:homocysteine methyltransferase (BHMT), which uses betaine as a methyl donor. The product of both these reactions is methionine. In the transsulfuration pathway, the enzyme cystathionine β-synthase (CBS) condenses homocysteine and serine to form cystathionine, which is then converted to cysteine by cystathionine γ-lyase (CGL). Both CBS and CGL require vitamin B_6. It is apparent from Fig. 1 that homocysteine metabolism is tightly linked to the methionine and folate cycles and that several key enzymes and cofactors are instrumental in regulating cellular homocys-

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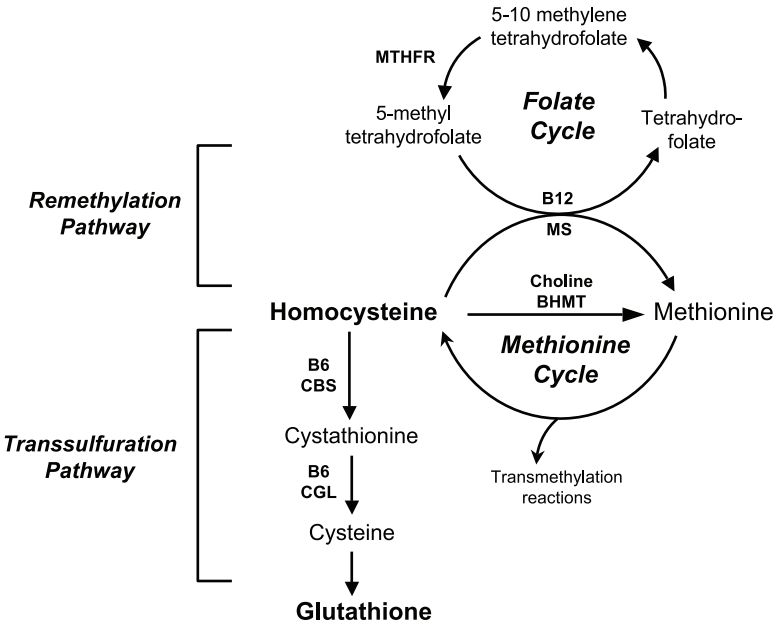

FIG. 1. Homocysteine metabolic pathways. In the methionine cycle, homocysteine is formed from methionine as a byproduct of transmethylation reactions. Homocysteine then either enters the transsulfuration pathway or is remethylated to methionine in the remethylation pathway. In the transsulfuration pathway, homocysteine is condensed with serine to form cystathionine by the enzyme cystathionine β-synthase (CBS). This reaction requires vitamin B_6 as a cofactor. Cystathionine is further metabolized to cysteine by the enzyme cystathionine γ lyase (CGL), which also requires vitamin B_6. Cysteine is a key substrate for the synthesis of glutathione. In the remethylation pathway, the enzyme methionine synthase (MS) transfers a methyl group from 5-methyltetrahydrofolate in a reaction that requires vitamin B_{12} as a cofactor. The remethylation pathway couples homocysteine metabolism to the folate cycle, because 5 methyltetrahydrofolate is derived from the activity of methylene tetrahydrofolate reductase (MTHFR). In the liver and kidney, homocysteine can undergo remethylation to methionine through an alternative reaction catalyzed by betaine/homocysteine methyltransferase (BHMT). The BHMT reaction uses betaine, a methyl donor derived from choline, rather than 5 methyltetrahydrofolate.

teine concentrations. Most cases of hyperhomocysteinemia are caused by defects in one of these enzymes or deficiencies of one or more of the vitamin cofactors.

It also is apparent from Fig. 1 that abnormalities in homocysteine metabolism may be linked to factors that affect the cellular redox state. For example, defects in homocysteine transsulfuration can lead to depletion of cysteine, which is a key substrate for the synthesis of the major intracellular antioxidant, glutathione. Another way in which homocysteine metabolism is linked to the cellular redox state is through the folate cycle. In addition to its role in homocysteine remethylation, folate participates in the regulation of several antioxidant reactions. Some forms of folate have been proposed to act as direct antioxidants (89). Finally, because it contains a free thiol group, homocysteine itself may influence the cellular redox state by undergoing autooxidation reactions (38, 61, 71) or mixed disulfide reactions with proteins or other thiols (42). Thus, several of the metabolic defects that cause hyperhomocysteinemia may directly or indirectly modulate cellular redox reactions.

ANIMAL MODELS OF HYPERHOMOCYSTEINEMIA

The most commonly used approach to induce experimental hyperhomocysteinemia in animals involves dietary interventions that affect the metabolism of homocysteine *via* the transsulfuration or remethylation pathways. Dietary deficiency of vitamin B_6 limits the metabolic flux of homocysteine through the transsulfuration pathway, whereas deficiency of vitamin B_{12} and/or folate impairs the remethylation of homocysteine to methionine. Hyperhomocysteinemia also can be induced by adding high amounts of methionine, or even homocysteine itself, to animal chow or drinking water to increase the metabolic flux through the methionine cycle. Combination diets, such as those containing high amounts of methionine along with low amounts of folate and/or vitamin B_{12}, are also commonly used. These approaches have been used in minipigs (64), monkeys (48, 49), rabbits (65, 67), and rats (8, 22, 54, 87). Another key factor that has a strong influence on homocysteine metabolism in animals is the dietary content of choline. Choline serves as a dietary source of betaine, which promotes homocysteine remethylation through the alternative BHMT reaction. Some standard animal chows contain limiting amounts of choline, which can increase susceptibility to hyperhomocysteinemia (68).

One limitation of dietary approaches to induce hyperhomocysteinemia is that the diets may affect levels of several metabolites in addition to homocysteine. For studies of vascular function, it may be difficult to distinguish responses caused by elevated homocysteine as opposed to other factors that are altered by the experimental diets. For example, folate-deficient diets may deplete plasma and tissue folate pools independent of their effects on homocysteine metabolism. Similarly, methionine-rich diets can influence other methionine-dependent reactions (such as transmethylation reactions; see Fig. 1) that may affect vascular end points. Both folate deficiency and methionine excess have been proposed to have adverse effects on vascular function that may be independent of elevated homocysteine (27, 82). Conversely, deficiency states for folate, vitamin B_6, and vitamin B_{12} are among the most common causes of hyperhomocysteinemia in humans (47), so these dietary models may have direct relevance to human vascular disease.

In the past decade or so, a number of genetic models of hyperhomocysteinemia have been generated in mice through the targeted deletion of genes encoding key enzymes of homocysteine metabolism. These include cystathionine β-synthase (*Cbs*) (93), methylene tetrahydrofolate reductase (*Mthfr*) (13), and

methionine synthase (*Mtr*) (73). Mice homozygous for targeted deletion of any of these genes generally do not survive past weaning. Some groups have been successful in weaning homozygous *Cbs*-deficient mice (63, 91). These mice have severe hyperhomocysteinemia (plasma tHcy >200 μM) but their suitability for studies of vascular disease is quite limited because of severe growth retardation. This limitation may be overcome in an interesting new transgenic mouse model in which an inducible human *CBS* transgene is expressed in homozygous *Cbs*-deficient mice (92), but the utility of this new mouse model for vascular studies has not yet been established.

For analysis of vascular phenotype, therefore, mice that are heterozygous for targeted deletions of *Cbs*, *Mthfr*, or *Mtr* have been used most frequently (16, 17, 19, 20, 24, 25, 29). When fed a standard rodent chow containing adequate amounts of choline, these heterozygous mice generally have relatively mild hyperhomocysteinemia (plasma tHcy of 6–9 μM, compared with 3–5 μM in their wild-type littermates). When fed diets that are high in methionine or low in folate or both, however, heterozygous *Cbs*, *Mthfr*, or *Mtr* mice can develop moderate to severe hyperhomocysteinemia (plasma tHcy of 10–100 μM) (13, 16, 17, 19, 20, 24, 25, 29, 45, 73, 93). As noted earlier, when using combined dietary and genetic approaches, it is important to keep in mind that some phenotypic effects may be mediated by dietary factors that are independent of homocysteine.

VASCULAR EFFECTS OF HYPERHOMOCYSTEINEMIA IN ANIMAL MODELS

Many distinct abnormalities of vascular structure and function have been described in animal models of hyperhomocysteinemia (Table 1). The vascular phenotypic effects of hyperhomocysteinemia observed in animal models include alterations of the endothelium and vascular muscle, altered mechanical properties of the vascular wall, accelerated development of thrombosis and atherosclerosis, and myocardial metabolic disease.

Endothelium

The first evidence for endothelial injury during hyperhomocysteinemia came from observations in baboons in the 1970s (36, 37). In these early studies, infusion of homocysteine or homocysteine thiolactone caused endothelial denudation and an increase in circulating endothelial cells. Several years later, in 1996, Matthias and co-workers (53) observed loss of aortic endothelium in rats fed high amounts of methionine. Some recent studies using rat and mouse models have found that hyperhomocysteinemia inhibits postinjury re-endothelialization of the carotid artery (54, 77). Many groups have reported abnormalities of endothelium-dependent vasomotor function in animals with hyperhomocysteinemia (45). The most common abnormality of vasomotor function observed during hyperhomocysteinemia is impaired relaxation of conduit vessels (such as the aorta, pulmonary, renal, or carotid arteries) or impaired dilatation of microvessels (such as those in the skeletal muscle, mesentery, or on the surface of the brain) in response to endothelium-dependent vasodilators.

The major mechanism by which hyperhomocysteinemia exerts its adverse effects on endothelial function is by diminishing relaxation responses that are mediated by endothelium-derived nitric oxide (NO) (16, 29, 43, 49, 79, 87). Endothelium-dependent vasodilators, such as acetylcholine, methacholine, thrombin, bradykinin, or shear stress, stimulate endothelial cells to produce NO through the calcium-dependent activation of endothelial nitric oxide synthase (eNOS or *Nos3*). NO then diffuses into the adjacent vascular muscle layer, causing its relaxation. With a model of diet-induced hyperhomocysteinemia, we found that monkeys fed a hyperhomocysteinemic diet had significantly decreased relaxation responses to acetylcholine in the carotid artery, and also in hindlimb resistance vessels, compared with monkeys fed a control diet (49). Subsequently, a similar degree of impairment of relaxation was observed in the aorta, carotid artery, or pulmonary artery of guinea pigs (79), rats (75), and mice (17, 29, 46) with hyperhomocysteinemia. Impaired dilatation or paradoxic vasoconstriction in response to endothelium-dependent dilators also has been demonstrated in mesenteric (29), cremasteric (42a), coronary (72, 75, 85), skeletal (3, 87), and cerebral (16, 19, 24) arterioles in hyperhomocysteinemic rats or mice.

In most of these studies, impaired relaxation or dilatation was seen selectively in response to endothelium-dependent stimuli, whereas no impairment was seen in response to exogenous NO donors (such as nitroprusside) or other endothelium-independent agonists. These observations suggest that the primary defect is related to decreased bioavailability of endothelium-derived NO rather than to an intrinsic defect in vascular muscle relaxation. In some vascular beds, such as the renal arterial system, hyperhomocysteinemia may also cause impaired bioavailability of other endothelium-derived vasodilators, including an endothelium-derived hyperpolarizing factor (22).

Altered vascular structure and mechanics

Structural abnormalities of the vascular wall have been observed in several animal models of hyperhomocysteinemia. In 1995, Rolland and colleagues (64) observed alterations in the structure of the medial layer of the abdominal aorta and coronary arteries of minipigs fed a methionine-rich diet. Histologic examination of these vessels revealed focal areas of smooth muscle hyperplasia, fragmentation of the internal elastic lamina, and disruption of elastic fibers. Similar abnormalities, including increased collagen deposition in the media and erosion of the internal elastic lamina, have been observed in the aortae of hyperhomocysteinemic rats and mice (53, 59). Hyperhomocysteinemia also has been reported to lead to accelerated formation of neointimal lesions after balloon injury of the carotid artery in rats (12, 15, 54, 58). These findings are consistent with clinical observations implicating hyperhomocysteinemia as a risk factor for increased carotid intima–media thickness (1, 52).

Our group measured the cross-sectional area (CSA) of maximally dilated cerebral arterioles on the pial surface of the brain of hyperhomocysteinemic mice (5). We found that hyperhomocysteinemia, induced by either heterozygous deficiency of the *Cbs* gene or a high-methionine diet, led to significant vascular hypertrophy, with a 25% increase in the CSA of the vascular wall. The increase in CSA was primarily caused by an increase in the medial content of collagen and elastin, which

Table 1. Vascular Phenotypes Observed in Hyperhomocysteinemic Animals

Phenotype	*Vascular bed*	*Animal model*	*References*
Endothelial denudation	Aorta	Baboons	36, 37
		Rats	53
Diminished re-endothelialization	Carotid artery	Rats	54
		Mice	77
Endothelial vasomotor dysfunction	Carotid artery	Monkeys	48, 49
		Rats	54
	Aorta	Rabbits	43
		Rats	75
		Mice	17, 29, 46
	Pulmonary artery	Guinea pigs	79
	Coronary artery	Dogs	72
		Rats	75, 85
	Skeletal muscle arterioles	Rats	3, 87
	Mesenteric arterioles	Rats	75
		Mice	29, 95
	Cremasteric artery	Mice	42a
	Pial arterioles	Mice	16, 19, 24
	Renal artery	Rats	22
Arterial hypertrophy or remodeling	Aorta	Minipigs	64
		Rats	53
		Mice	59
	Coronary artery	Minipigs	64
	Pial arterioles	Mice	6
Increased neointima formation after injury	Carotid artery	Rats	12, 15, 54, 58
		Mice	77
Altered vascular mechanics	Carotid	Rats	74
	Pial arterioles	Mice	57, 59, 76
		Mice	6
Altered vascular permeability	Carotid	Rats	74
		Mice	57, 76
	Pial venules	Mice	50
Accelerated thrombosis	Carotid artery	Mice	20, 98
Increased atherosclerosis	Aorta	Rabbits	80
		Mice	82, 91, 102, 103
Myocardial dysfunction	Heart	Dogs	72
		Rats	8

suggests that the vascular hypertrophy of hyperhomocysteinemia is related to altered regulation of the extracellular matrix. Ovechkin *et al*. (59) observed a similar expansion of the extracellular matrix, as well as disruption and dysregulation of elastin fibers, in the aortae of hyperhomocysteinemic mice. These authors suggested that dysregulation of the extracellular matrix in hyperhomocysteinemia may be caused by the induction and activation of matrix metalloproteinases (MMPs), perhaps through a redox mechanism.

In addition to its association with vascular hypertrophy and remodeling, hyperhomocysteinemia has been found in some studies to produce alterations of the mechanical properties of the vascular wall. Increased arterial stiffness has been documented in rat and mouse models of hyperhomocysteinemia (57, 74, 76). In contrast, some other studies of hyperhomocysteinemic mice have demonstrated an increase in vascular compliance, or elasticity, in vessels with increased elastin content (6, 59). The pathophysiologic consequences of these alterations in vascular mechanics are not known. It is possible that these alterations may further exacerbate the adverse effects of endothelial dysfunction induced by hyperhomocysteinemia in some vascular beds. Alternatively, the increase in vascular compliance may represent a compensatory protective response to hyperhomocysteinemia.

Thrombosis

Hyperhomocysteinemia is a clinical risk factor for both arterial and venous thrombosis (45). In animal models, hyperho-

mocysteinemia does not usually cause spontaneous thrombosis, but hyperhomocysteinemic mice have been found to have increased susceptibility to experimental thrombosis. With a laser-induced photochemical injury method, we demonstrated that hyperhomocysteinemic mice have an accelerated thrombotic response to endothelial injury in the carotid artery (20). Complete thrombotic occlusion of the carotid artery occurred 50% faster in mice fed a high-methionine/low-folate diet than in those fed a control diet. We also examined the effect of hyperhomocysteinemia on thrombotic susceptibility in apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice, which have chronic hypercholesterolemia (98). We found that the occlusive thrombotic response to carotid artery injury was accelerated in *Apoe*$^{-/-}$ mice fed either a high-fat diet or a hyperhomocysteinemic diet. The *Apoe*$^{-/-}$ mice fed the high-fat diet had plasma levels of total cholesterol that were $>$16 m*M*, and those fed the hyperhomocysteinemic diet had plasma tHcy levels of 5–12 μM. These observations suggest that moderate hyperhomocysteinemia produces a prothrombotic effect in mice that is similar to that of severe hypercholesterolemia.

Several potential mechanisms may contribute to accelerated thrombosis in hyperhomocysteinemic animals. The vascular endothelium plays an important role in preventing interactions of blood cells with the vessel wall and helps in maintaining a nonthrombogenic luminal surface. Endothelium-derived NO is a potent inhibitor of neutrophil adhesion and platelet activation. Therefore, endothelial dysfunction with loss of endothelium-derived NO is a plausible mechanism for accelerated thrombosis in hyperhomocysteinemia. Mice with complete genetic deficiency of the gene for endothelial NO synthase (*Nos3*) do not exhibit enhanced susceptibility to experimental thrombosis, however, which suggests that chronic loss of endothelium-derived NO is not in itself sufficient to produce accelerated thrombosis in mice (20, 41, 60).

Another important endothelial product that has a critical anticoagulant function is thrombomodulin. Thrombomodulin is a glycoprotein that is expressed on the luminal surface of the endothelium (94). It is a key cofactor for the activation of anticoagulant protein C by thrombin. We have demonstrated that thrombomodulin-dependent activation of protein C by thrombin is reduced in the carotid artery of monkeys fed a high-methionine/low-folate diet (49) and also in the aortae of heterozygous *Cbs*-deficient mice fed hyperhomocysteinemic diets (17, 20). The reduced capacity of thrombomodulin to activate protein C may predispose to thrombosis in mice. Interestingly, thrombomodulin is highly susceptible to oxidative inactivation (33), which suggests a possible redox-dependent mechanism of accelerated thrombosis in hyperhomocysteinemia. This possible mechanism has not yet been tested directly in animal models.

Atherosclerosis

As noted earlier, hyperhomocysteinemia produces several distinct alterations in vascular structure and function that might be expected to influence the development of atherosclerosis. In the absence of hyperlipidemia, however, none of the animal models of hyperhomocysteinemia has been observed to develop spontaneously true atherosclerotic lesions. Conversely, hyperhomocysteinemia has been observed to potentiate the development of advanced atherosclerosis in hyperlipidemic *Apoe*$^{-/-}$ mice.

Hofmann *et al*. (39) reported that *Apoe*$^{-/-}$ mice fed a hyperhomocysteinemic diet developed atherosclerotic lesions in the aortic sinus that were of greater size and complexity than those seen in *Apoe*$^{-/-}$ mice fed normal chow (39). Several other studies, by using a variety of dietary and genetic approaches to induce hyperhomocysteinemia in *Apoe*$^{-/-}$ mice, confirmed and extended the observations of Hofmann (91, 102). One study suggested that a high dietary intake of methionine is atherogenic in *Apoe*$^{-/-}$ mice, even in the absence of significant hyperhomocysteinemia (82). Hyperhomocysteinemia induced by a high-methionine diet also has been shown to accelerate atherosclerosis in rabbits (80). If confirmed, these findings might suggest that atherosclerosis is not influenced by homocysteine itself but by another factor related to methionine.

ROLE OF REDOX REACTIONS IN THE VASCULAR PATHOPHYSIOLOGY OF HYPERHOMOCYSTEINEMIA

Redox reactions resulting in increased vascular levels of ROS or RNS may play an important role in generating the vascular phenotype of hyperhomocysteinemia (Fig. 2). The emerging evidence suggests a major role of redox reactions in mediating endothelial dysfunction, particularly the loss of endothelium-derived NO, during hyperhomocysteinemia. Good evidence exists for the increased generation of both ROS, such as superoxide and hydrogen peroxide, and RNS, such as peroxynitrite, in vascular tissues of hyperhomocysteinemic animals. Redox reactions also may play a role in the development of vascular hypertrophy and remodeling in hyperhomocysteinemia. The pathophysiologic role of redox reactions is less clearly defined for some of the other vascular phenotypes of hyperhomocysteinemic animals, such as thrombosis and atherosclerosis.

The role of redox reactions in the endothelial dysfunction of hyperhomocysteinemic animals has been examined by using both pharmacologic and genetic approaches. In some animal models of hyperhomocysteinemia, antioxidant compounds such as ascorbic acid or oxothiazolidine-4-carboxylic acid (a cysteine donor) have been found to protect the endothelium from vasomotor dysfunction (72, 95). Lang and colleagues (43) showed impaired endothelium-dependent relaxation of isolated rings of rabbit aorta in the presence of homocysteine. This response was restored toward normal by preincubating the aortic rings with tiron, an intracellular free radical scavenger. These findings were later confirmed in an *in vivo* study of cerebral arterioles in which impaired endothelium-dependent vasodilatation in mice fed a high-methionine diet was restored to normal in the presence of tiron (16). These data suggest that an oxygen free radical, such as superoxide or other ROS that are scavenged by tiron, may be directly responsible for endothelial dysfunction in hyperhomocysteinemia.

Eberhardt and colleagues (29) showed that addition of pegylated superoxide dismutase (PEG-SOD), an antioxidant enzyme that converts superoxide to hydrogen peroxide, to the mesenteric bed of heterozygous *Cbs*-deficient mice caused reversal of vasoconstrictor responses to methacholine. These findings suggest that endothelium-dependent vasomotor responses were impaired by hyperhomocysteinemia through a superoxide-dependent mechanism in this model. Other groups of investigators reported similar protective effects PEG-SOD in preparations of pulmonary artery, aorta, or skeletal muscle ar-

terioles from hyperhomocysteinemic guinea pigs (79), rats (3, 78) and rabbits (69).

In addition to pharmacologic approaches, genetic approaches have been taken to attempt to define the role of specific redox reactions in mediating endothelial dysfunction in hyperhomocysteinemic animals. Weiss and colleagues (96) crossbred heterozygous *Cbs*-deficient mice to mice overexpressing glutathione peroxidase-1 (*Gpx1*), an intracellular antioxidant enzyme that hydrolyzes hydrogen peroxide and lipid peroxides. They found that overexpression of *Gpx1* corrected the abnormal endothelium-dependent vasodilator responses in *Cbs*-deficient mice (96). Taking a complementary approach, our group found that mice deficient in *Gpx1* have increased susceptibility to hyperhomocysteinemia-induced endothelial dysfunction (18). More recently, we found that genetic deficiency of SOD also predisposes to endothelial dysfunction in hyperhomocysteinemic mice (Dayal and Lentz, unpublished observations).

Taken together, these observations from multiple laboratories suggest that both superoxide and peroxides are important mediators of endothelial dysfunction in hyperhomocysteinemia. A key pathophysiologic mechanism of endothelial dysfunction likely involves the oxidative inactivation of endothelium-derived NO through its reaction with superoxide (see Fig. 2). The product of this reaction, peroxynitrite ($ONOO^-$), may cause further inhibition of NO production through the direct inhibition and/or uncoupling of eNOS (105). In support of this mechanism, increased levels of nitrotyrosine, an oxidative marker of peroxynitrite generation, have been observed in the aortae of hyperhomocysteinemic mice (29, 85). It also has been demonstrated that abnormal endothelium-dependent vasomotor responses in hyperhomocysteinemic rats can be reversed by preincubation with urate, a peroxynitrite scavenger (3).

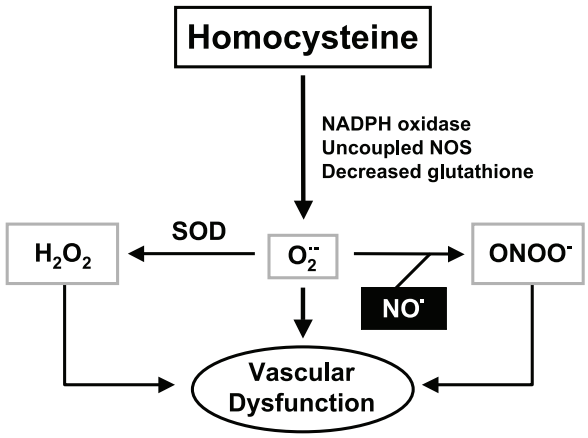

FIG. 2. Reactive oxygen and nitrogen species (ROS and RNS) in homocysteine-induced vascular dysfunction. Likely sources of homocysteine-induced ROS and RNS in vascular tissue include NADPH oxidase, uncoupled nitric oxide synthase (NOS), and depletion of intracellular glutathione. This leads to vascular dysfunction through the increased generation of superoxide ($O_2^{\cdot-}$). Superoxide can also be converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) or react with nitric oxide ($NO^{\cdot}$) to produce peroxynitrite ($ONOO^-$), causing further vascular dysfunction.

Redox reactions also may play a causative role in the development of vascular hypertrophy in hyperhomocysteinemia. In cerebral arterioles, compelling evidence indicates that deficiency of endothelium-derived NO can cause vascular hypertrophy (7). Good data suggest that cerebral vascular hypertrophy can be caused by superoxide, because genetic deficiency of SOD leads to a marked increase in the CSA of pial arterioles (5). These observations suggest the possibility that vascular hypertrophy in hyperhomocysteinemia may be caused by superoxide-mediated inactivation of endothelium-derived NO. Superoxide may also promote hypertrophy by activating MMPs, leading to remodeling of the extracellular matrix of the vascular wall (59). These possibilities could be tested by determining whether mice with increased or decreased SOD activity are protected or sensitized, respectively, to hyperhomocysteinemia-induced vascular hypertrophy.

The role of redox reactions in mediating the prothrombotic effects of hyperhomocysteinemia is less well documented. It is readily apparent, however, that redox reactions have many potential effects on platelets and other vascular cells involved in the initiation and propagation of pathologic thrombi. Thrombotic processes that may be altered by redox reactions include inhibition of platelet activation by NO, induction of procoagulant tissue factor, and oxidative inactivation of the endothelial anticoagulant, thrombomodulin. We have found that accelerated thrombosis in hyperhomocysteinemic mice is associated with increased superoxide production and decreased thrombomodulin activity (20). A mechanistic link between these factors, however, has not been definitively established.

Because it is an aminothiol, homocysteine can undergo redox and disulfide exchange reactions that can lead to the chemical modification of proteins through S-thiolation linkages (42). In its thiolactone form, homocysteine also can modify proteins through N-acylation reactions (62). Such modifications have the potential to modify protein function directly or to alter protein stability or expression by influencing protein conformation. At least three proteins that have been identified as targets of S-thiolation or N-acylation by homocysteine may contribute to a prothrombotic state: (a) factor V (84), (b) annexin II (34), and (c) fibrinogen (66). Homocysteine-modified fibrinogen may be particularly prothrombotic, because it has been demonstrated in a rabbit model that fibrin clots formed in hyperhomocysteinemic plasma are unusually resistant to lysis (28, 65, 67).

Atherosclerosis is a complicated process that is known to involve multiple cellular stress responses, including oxidative stress, endoplasmic reticulum (ER) stress, and inflammatory pathways (104). It is not known whether hyperhomocysteinemia accelerates atherosclerosis through its effects on redox reactions or through other mechanisms. Some studies detected increased levels of markers of ROS or RNS in the plasma and atherosclerotic lesions of rabbits and mice fed hyperhomocysteinemic diets (80, 104). Other studies suggested that hyperhomocysteinemia may accelerate atherosclerosis by activating ER-stress pathways (101, 104). This hypothesis is based on indirect evidence, however, and no direct links between redox reactions or ER stress and accelerated atherosclerosis in hyperhomocysteinemia have been established. This will be an exciting area for future investigation.

SOURCES OF VASCULAR ROS AND RNS IN HYPERHOMOCYSTEINEMIA

The major mechanisms that have been proposed for the generation of vascular ROS and RNS in hyperhomocysteinemia are illustrated in Fig. 3. The relative importance of each mechanism is likely to vary in different vascular beds and also may vary in different animal species.

NADPH oxidase

Vascular NADPH oxidases have been identified as major sources of superoxide and other ROS in a variety of pathologic conditions, including hypertension, diabetes, and atherosclerosis (10, 51, 56). NADPH oxidases are multisubunit enzymes that contain both cytosolic and membrane components (44). Increased expression of the NADPH oxidase membrane subunits *Nox1* and *Nox4* has been detected in the aortae of hyperhomocysteinemic rats and mice (20, 86). Interestingly, upregulation of several cytosolic subunits of NADPH oxidase, including $p22^{phox}$, rac-1, and $p47^{phox}$, has been observed in cardiac tissue of hyperhomocysteinemic animals (8, 72).

Additional evidence for a role of NADPH oxidase in the generation of vascular ROS in hyperhomocysteinemia was obtained by Ungvari and colleagues (86). These investigators detected elevated levels of vascular ROS by both dihydroethidium (DHE) fluorescence and lucigenin-enhanced chemiluminescence in the coronary arteries of hyperhomocysteinemic rats. The generation of ROS in these vessels was largely blocked by PEG-SOD, which suggests that superoxide was the major ROS detected, and also by apocynin, a relatively specific inhibitor of NADPH oxidase. In a subsequent study in hyperhomocysteinemic mice, we observed a similar increase in DHE fluorescence in the carotid artery that was also inhibited by PEG-SOD and apocynin (16). Two very recent studies in dog and rabbit models have provided more direct evidence for the role of NADPH oxidase in the endothelial dysfunction of hyperhomocysteinemia. In these studies, impaired relaxation responses in the coronary artery or aorta were restored toward normal in the presence of apocynin (69, 72). Together, these findings suggest that hyperhomocysteinemia leads to increased vascular superoxide production through the upregulation and activation of NADPH oxidase, and that ROS generated by NADPH oxidase contribute to the vascular phenotype.

At least three possible mechanisms may account for the upregulation of NADPH oxidase activity in hyperhomocysteinemia (see Fig. 3). First, the inflammatory cytokine tumor necrosis factor-α (TNF-α), which is known to activate NADPH oxidase in cultured rat aortic smooth muscle cells and human pulmonary endothelial cells (21, 32), has been found to be upregulated in the coronary artery of hyperhomocysteinemic rats (86). The upregulation of TNF-α paralleled the increased expression of *Nox1* and the increased activity of NADPH (86). These findings suggest that TNF-α may be a mediator of vascular NADPH activation in hyperhomocysteinemia. Second, activation of endothelial surface receptors, such as the proteolytically activated receptor-4 (PAR4), may upregulate NADPH oxidase activity. It has been demonstrated that homocysteine induces increased expression PAR4 in conjunction with *Nox1* in cultured microvascular endothelial cells (83). Third, both the rac-1 and *Nox4* genes contain CpG islands in their promoters, which raises the intriguing possibility that upregulation of these genes could be mediated by hyperhomocysteinemia-induced DNA hypomethylation (25).

eNOS/iNOS uncoupling

In blood vessels, NO is normally produced by endothelial NO synthase (eNOS or *Nos3*). eNOS is expressed constitutively in vascular endothelium, and it is activated in response to normal homeostatic stimuli. A second, inducible NOS gene (iNOS or *Nos2*) can be expressed in vascular smooth muscle cells, and also in endothelium, under pathologic conditions such as ath-

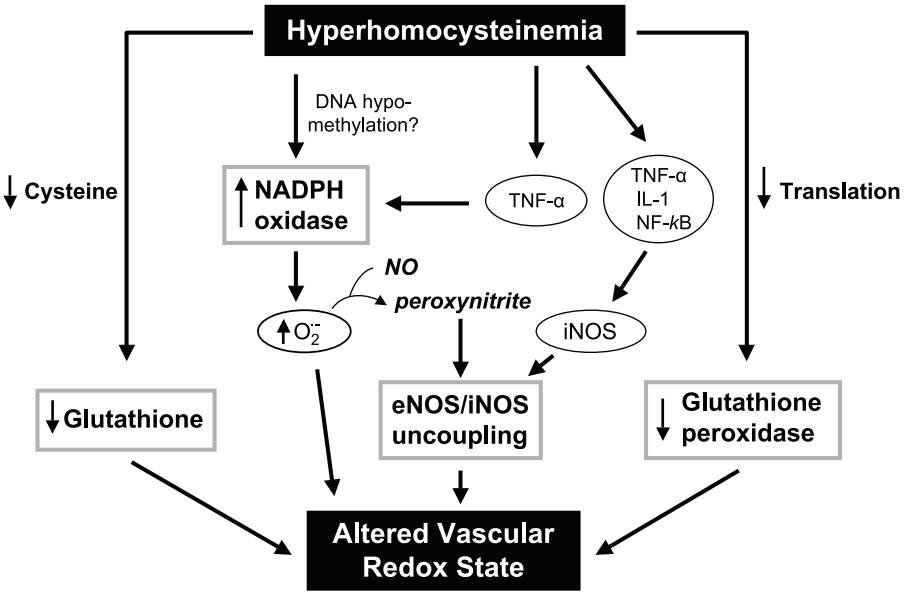

FIG. 3. Possible mechanisms of altered vascular redox state in hyperhomocysteinemia. Hyperhomocysteinemia may alter the vascular redox state through at least four different mechanisms: (a) upregulation and/or activation of NADPH oxidase, (b) uncoupling of eNOS and/or iNOS, (c) downregulation of expression of glutathione peroxidase, and (d) depletion of intracellular glutathione.

erosclerosis, diabetes, and other inflammatory disorders (4, 9, 23, 40). Inflammatory cytokines such as TNF-α, interleukin-1, and NF-κB, which have been shown to be elaborated during hyperhomocysteinemia in some animal models (39, 86, 99), may contribute to the upregulation of iNOS. Increased expression of iNOS has been observed in cultured endothelial and smooth muscle cells treated with homocysteine (83, 97) and also in coronary arteries and kidneys of hyperhomocysteinemic rats (86, 99).

Under some pathologic conditions, both eNOS and iNOS can become "uncoupled," a circumstance in which the normal flow of electrons within the enzyme is diverted so that superoxide, rather than NO, becomes the predominant reaction product (11). Uncoupling of eNOS may be caused by oxidation of its cofactor, tetrahydrobiopterin, perhaps through the action of peroxynitrite (105). Evidence for eNOS uncoupling and reduced availability of tetrahydrobiopterin in the presence of homocysteine was obtained from the studies in cultured endothelial cells (81, 100). These findings are consistent with a study in hyperhomocysteinemic rats, which found that impaired endothelium-dependent vasomotor responses in the aorta could be reversed by preincubation with tetrahydrobiopterin (26) . Additionally, we and others showed that increased DHE fluorescence in the carotid and coronary arteries of hyperhomocysteinemic rats or mice can be inhibited by NOS inhibitors (16, 86). Together, these studies indicate that uncoupling of eNOS and/or iNOS may contribute to the altered vascular redox state in hyperhomocysteinemia. The specific roles of eNOS *versus* iNOS, as well as the relative importance of NOS uncoupling compared with NADPH oxidase activation, remain to be determined.

Glutathione peroxidase

Glutathione peroxidase-1 (Gpx1) is a major intracellular antioxidant enzyme that catalyzes the hydrolysis of hydrogen peroxide and lipid peroxides (2). By using bovine aortic endothelial cells in culture, Loscalzo and colleagues (88) showed nearly a decade ago that homocysteine causes decreased activity of Gpx1. They further showed that the decrease in Gpx1 activity was not due to direct inhibition by homocysteine, but was instead attributable to decreased expression of Gpx1. In more recent work, these investigators demonstrated that homocysteine inhibits Gpx1 expression in cultured endothelial cells by inhibiting translation of its mRNA (35). A decrease in glutathione peroxidase activity in hepatic tissues of hyperhomocysteinemic mice has also been reported (95). The posttranscriptional downregulation of Gpx1 expression in hyperhomocysteinemia may lead to increased vascular levels of hydrogen peroxide and other ROS.

Glutathione

Glutathione is an important thiol redox buffer in cells that helps maintain a reduced environment within the cytoplasm. The synthesis of glutathione utilizes cysteine generated from homocysteine through the transsulfuration pathway (see Fig. 1). Hyperhomocysteinemia caused by severe defects in transsulfuration (such as *Cbs* deficiency or deficiency of vitamin B_6) might therefore be expected to lead to decreased intracellular levels of glutathione and a decreased cellular thiol-buffering capacity. The oxidant stress of hyperhomocysteinemia, with increased levels of ROS and RNS, may further compromise the thiol-buffering capacity by decreasing the GSH/GSSG ratio. Elevation of homocysteine also might deplete glutathione through direct thiol oxidation reactions (42). Decreased levels of cysteine and glutathione have been found in the liver and kidney of homozygous *Cbs*-deficient mice (90). It is not known whether depletion of glutathione occurs to a significant extent in heterozygous *Cbs*-deficient mice or other animals with moderate hyperhomocysteinemia, however, and the relative importance of glutathione deficiency *versus* other causes of increased ROS and RNS in vascular tissue has not been examined in any of these models.

PERSPECTIVE

Our current understanding of the molecular mechanisms mediating the adverse vascular effects of hyperhomocysteinemia is largely derived from work with animal models. Much of this work implicates redox reactions as major contributors to the vascular phenotype of hyperhomocysteinemia. Evidence for high levels of both ROS and RNS has been found in both large arteries and microvessels of hyperhomocysteinemic animals. Sources of ROS and RNS in vascular tissue likely include NADPH oxidase and uncoupled eNOS or iNOS. Downregulation of glutathione peroxidase and depletion of intracellular glutathione may contribute to further increases in the vascular levels of ROS and RNS, particularly in animals with severe hyperhomocysteinemia. The role of redox reactions appears to be especially important in mediating the adverse effects of hyperhomocysteinemia on the endothelium, leading to loss of endothelium-derived NO and vasomotor dysfunction. Redox reactions that increase ROS and decrease NO also may be key factors in the development of vascular hypertrophy in hyperhomocysteinemic animals. Although a strong rationale supports the hypothesis that redox reactions also play a role in mediating other vascular phenotypic effects of hyperhomocysteinemia, such as thrombosis and atherosclerosis, direct evidence to support this hypothesis is currently lacking.

Future studies are needed to define the relative roles of altered redox reactions *versus* other factors, including ER stress and inflammatory mediators such as TNF-α, in the prothrombotic and atherogenic phenotypes of hyperhomocysteinemia. Other important areas for future investigation include studies to confirm the importance of ROS and RNS in mediating vascular hypertrophy and neointimal proliferation in hyperhomocysteinemia. Finally, still much work remains to be done to define the exact role of homocysteine, as opposed to related metabolites such as methionine or various products of transmethylation reactions, as upstream activators of redox reactions.

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ABBREVIATIONS

Apoe, murine apolipoprotein E gene; BHMT, betaine/homocysteine methyltransferase; CBS or *Cbs,* cystathionine β-synthase; CGL, cystathionine γ-lyase; CSA, cross-sectional area; ER, endoplasmic reticulum; eNOS or *Nos3,* endothelial nitric oxide synthase; Gpx1, glutathione peroxidase-1; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; iNOS or *Nos2,* inducible nitric oxide synthase; MMP, metalloproteinase; MTHFR or *Mthfr,* methylene tetrahydrofolate reductase; *Mtr,* murine methionine synthase gene; NF-κB, nuclear factor kappa-B; NO, nitric oxide; $ONOO^-$, peroxynitrite; PEG-SOD, pegylated superoxide dismutase; ROS, reactive oxygen species; RNS, reactive nitrogen species; TNF-α, tumor necrosis factor-α.

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