REVIEW ARTICLE

Folic Acid Says NO to Vascular Diseases

Undurti N. Das, MD, FAMS

From EFA Sciences LLC, Norwood, Massachusetts, USA

OBJECTIVES: The possible link between folic acid or folate and tetrahydrobiopterin (H_4B), vitamin C, polyunsaturated fatty acids (PUFAs), and nitric oxide (NO), which may explain the beneficial actions of these nutrients in various vascular conditions, was investigated.

METHODS: The literature pertaining to the actions of folic acid/folate, H_4B, vitamin C, PUFAs, and NO was reviewed.

RESULTS: Impaired endothelial NO (eNO) activity is an early marker for cardiovascular disease. Most risk factors for atherosclerosis are associated with impaired endothelium-dependent vasodilatation due to reduced NO production. Folate not only reduces plasma homocysteine levels but also enhances eNO synthesis and shows anti-inflammatory actions. It stimulates endogenous H_4B regeneration, a cofactor necessary for eNO synthesis, inhibits intracellular superoxide generation, and thus enhances the half-life of NO. H_4B in turn enhances NO generation and augments arginine transport into the cells. Folic acid increases the concentration of ω-3 PUFAs, which also enhance eNO synthesis. Vitamin C augments eNO synthesis by increasing intracellular H_4B and stabilization of H_4B. Insulin stimulates H_4B synthesis and PUFA metabolism, suppresses the production of proinflammatory cytokine tumor necrosis factor-α and superoxide anion, and enhances NO generation. The ability of folate to augment eNO generation is independent of its capacity to lower plasma homocysteine levels.

CONCLUSIONS: The common mechanism by which folic acid, H_4B, vitamin C, ω-3 fatty acids, and L-arginine bring about their beneficial actions in various vascular diseases is by enhancing eNO production. Hence, it remains to be determined whether a judicious combination of folic acid, vitamins B12, B6, and C, H_4B, L-arginine, and ω-3 fatty acids in appropriate amounts may form a novel approach in the prevention and management of various conditions such as hyperlipidemias, coronary heart disease, atherosclerosis, peripheral vascular disease, and some neurodegenerative conditions. *Nutrition* 2003;19: 686–692. ©Elsevier Inc. 2003

KEY WORDS: homocysteine, coronary heart disease, folic acid, vitamin C, polyunsaturated fatty acids, nitric oxide, L-arginine, tetrahydrobiopterin, superoxide anion, lipid peroxidation

INTRODUCTION

Inadequate intake of folic acid and, to a lesser extent, vitamins B6 and B12 increases homocysteine levels. Hyperhomocystinemia is an independent risk factor for atherosclerosis, coronary heart disease (CHD), and venous thromboembolism.[1–3] Conversion of homocysteine to methionine requires methyl folic acid as the methyl donor and vitamin B12 as a cofactor. Hence, hyperhomocystinemia might be associated with low concentrations of methionine, methyl folic acid, or vitamin B12. Deficiency of any one of these components could lead to vascular disease. Genetic polymorphisms of methylene tetrahydrofolic acid reductase enzyme, which catalyzes methyl folic acid production, could also play a role in vascular diseases. However, the C677T methylene tetrahydrofolic acid reductase polymorphism, although associated with hyperhomocystinemia, is not a risk factor for venous thromboembolism.[4] Homocysteine concentrations depend on a series of intracellular metabolic reactions in which folate (in this review, *folate* refers to the natural form in the human body and *folic acid* refers to the synthetic compound used in supplements) acts as a substrate and vitamin B12 serves as a coenzyme (see Fig. 1 for homocysteine methylation pathway), and it is believed that adequate supplementation of these two vitamins is sufficient to lower levels of homocysteine and thus avert its adverse effect. However, recent studies have suggested that the benefits of folic acid are not limited to homocysteine metabolism. Folic acid appears to interact with nitric oxide (NO) metabolism, enhance the availability of tetrahydrobiopterin (H_4B), reduce superoxide anion generation, and, hence, improve endothelial dysfunction.

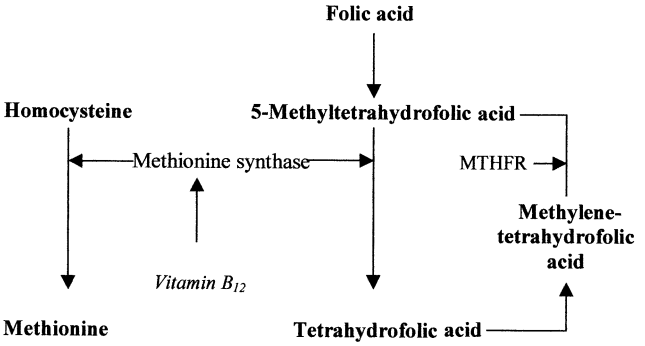

FIG. 1. Metabolism of homocysteine. MTHFR, methylene tetrahydrofolic acid reductase.

Homocysteine is a sulfur-containing amino acid formed during the metabolism of methionine. Once formed, homocysteine may undergo remethylation to methionine in a reaction catalyzed by methylene tetrahydrofolic acid homocysteine methyltransferase (methionine synthetase), which uses methyl tetrahydrofolic acid as a methyl donor and cobalamin as an essential cofactor.[5] Homo-

Correspondence to: Undurti N. Das, MD, FAMS, EFA Sciences LLC, 1420 Providence Highway, Suite 266, Norwood, MA 02062, USA. E-mail: undurti@hotmail.com or undurti@efasciences.com

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cysteine also enters the transsulfuration pathway when cysteine synthesis is required or in the presence of excess methionine for which pyridoxal 5′-phosphate is needed. Several genetic disorders can cause hyperhomocystinemia, which in turn produces atherothrombotic vascular disease that is frequently fatal in childhood and adolescence.[6] Severe hyperhomocystinemia is rare, although mild hyperhomocystinemia occurs in approximately 5% to 7% of the general population compared with the rarity of genetic disorders causing hyperhomocystinemia (the prevalence being 1:200 000 births).[7] Hyperhomocystinemia is found up to 40% of individuals with cerebrovascular, coronary, or peripheral vascular diseases.[8] Recent studies have shown that an increased plasma homocysteine concentration is the result of vitamin deficiencies, specifically folic acid, pyridoxine, and cobalamin. Supplementation of these vitamins have reduced or even normalized elevated plasma homocysteine levels.[9,10] In most clinical studies, measurement of total plasma homocysteine have included homocysteine, mixed disulfides involving homocysteine, homocysteine thio lactone, free homocysteine, and protein-bound homocysteine. Protein-bound homocysteine (i.e., disulfide linked) accounts for 70% to 80% of the total pool of homocysteine. Normal total plasma homocysteine concentrations have ranged from 5 to 15 μM/L in the fasting state.[7] On the basis of concentrations measured during fasting, hyperhomocystinemia is classified as moderate when homocysteine concentration ranges from 15 to 30 μM/L, intermediate when it ranges from 30 to 100 μM/L, and severe when it is above 100 μM/L.[7]

HOMOCYSTEINE, OXIDANT STRESS, AND NO

Homocysteine is believed to exert its effects through a mechanism involving oxidative damage.[11] Homocysteine is readily oxidized as a consequence of auto-oxidation leading to the formation of homocystine, homocysteine-mixed disulfides, and homocysteine thio lactone. During oxidation of the sulfhydryl group, superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) are generated, which account for the endothelial cytotoxicity of homocysteine.[11] These free radicals initiate lipid peroxidation, which oxidize low-density lipoprotein. Under normal conditions, endothelium is antithrombotic in nature. This is due to its ability to secrete prostacyclin and NO. Homocysteine converts the normal antithrombotic endothelium to a more prothrombotic phenotype by increasing factor V and factor XII activity, decreasing protein C activation, inhibiting thrombomodulin expression, inducing tissue factor expression, suppressing heparan sulfate expression, reducing the binding of tissue-type plasminogen activator to its endothelial cell receptor Annexin II, and reducing the production of NO and prostacyclin, events that lead to the generation of thrombin and facilitates thrombotic tendency.[11–13] Normal endothelial cells detoxify homocysteine by releasing NO or a related *S*-nitroso thiol, which in turn leads to the formation of *S*-nitroso-homocysteine,[14] a potent vasodilator and platelet anti-aggregator. This *S*-nitrosation of homocysteine (thus forming *S*-nitroso-homocysteine) attenuates sulfhydryl-dependent generation of H_2O_2. However, continued exposure of endothelium to homocysteine compromises the production of adequate amounts of NO that ultimately leads to unopposed homocysteine-mediated injury to the endothelium and initiation of atherosclerosis and/or thrombus formation or acceleration of existing atherosclerosis. Homocysteine attenuates the antithrombotic action of endothelium by enhancing the production of $O_2^{-\bullet}$ (generated during its auto-oxidation), which in turn inactivates NO. Normally, a balance is maintained between NO and $O_2^{-\bullet}$. Hence, measures designed to enhance NO production can abrogate this action of homocysteine and restore the antithrombotic actions of endothelium.

In addition, homocysteine inhibits glutathione peroxidase (GP) activity in vitro and reduces the steady-state mRNA levels for the intracellular isoform of GP in endothelial cells. GP catalyzes the reduction of H_2O_2 and lipid peroxides to their corresponding alcohols and thus prevents inactivation of NO. Inhibition of GP activity by homocysteine is one mechanism by which homocysteine produces its vascular toxicity because this is a property that is not shared by other biologic thiols.[15] This is the reason cysteine, an amino acid present in plasma at a concentration at least three- to four-fold greater than that of homocysteine, can generate $O_2^{-\bullet}$ during auto-oxidation without being toxic, simply because it cannot inhibit the activity and formation of GP. Further, endothelial NO (eNO) inhibits smooth muscle cell proliferation and migration. Homocysteine also induces smooth muscle cell migration and proliferation by increasing cyclin D1 and cyclin A mRNA expression[16] and inhibits NO production, which contribute to its proatherosclerotic and prothrombotic actions. Homocysteine upregulates vascular cell adhesion molecule-1 expression in human aortic endothelial cells and enhances monocyte adhesion. Cyclooxygenase inhibitors can completely abrogate homocysteine-induced monocyte adhesion, whereas scavenging reactive oxygen species and elevation of NO causes only partial inhibition.[17] Compared with control subjects, hyperhomocystinemic individuals showed elevated levels of the CXC chemokines: epithelial neutrophil-activating peptide-78 and growth-regulated oncogene-α. Folic acid treatment not only normalizes homocysteine levels but also reduces oxidized low-density lipoprotein–stimulated release of growth-regulated oncogene-α, epithelial neutrophil-activating peptide-78, interleukin-8, and CC chemokines monocyte chemoattractant peptide-1 and RANTES in peripheral blood mononuclear cells from these individuals. Oxidized low-density lipoprotein–induced release of epithelial neutrophil-activating peptide-78 by peripheral blood mononuclear cells from control subjects was significantly reduced when cells were incubated with folic acid.[18] These results support the idea that proinflammatory actions of homocysteine are mediated by prostaglandins. In this context, it is interesting to note that low circulating levels of vitamin B6 have been associated with higher C-reactive protein levels independent of plasma homocysteine levels.[19] The fact that rheumatoid arthritis is associated with reduced vitamin B6 levels suggests that this vitamin may have anti-inflammatory actions. Vitamin B6 enhances the production of prostaglandin E_1, a potent vasodilator, platelet anti-aggregator, and anti-inflammatory eicosanoid.[20–22] Thus, vitamin B6 lowers the concentrations of homocysteine and has anti-inflammatory actions.

Human umbilical vein endothelial cells, when exposed to homocysteine, have enhanced activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase enzyme, the rate-limiting enzyme in the synthesis of cholesterol. The cell-permeable superoxide dismutase (SOD) mimetic, manganese tetrakis-(4-benzoic acid) porphyrin [Mn-TBAP], can reverse homocysteine-induced expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity; 20, 50, and 100 μM/L of homocysteine resulted in 22.2 $\pm$ 7.3%, 39.5 $\pm$ 1.2%, and 50.4 $\pm$ 6.8% increases, respectively, in the total cellular cholesterol content of human umbilical vein endothelial cells. Simvastatin, an 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, reduced cellular cholesterol content and prevented homocysteine-induced suppression of NO production by human umbilical vein endothelial cells in a dose-dependent manner.[23] This suggests that homocysteine interacts directly with cholesterol metabolism, which could be one mechanism by which it participates in the pathogenesis of atherosclerosis.

FOLATE/FOLIC ACID, H_4B, OXIDANT STRESS, AND ENO

Folic acid lowers homocysteine and improves endothelial function. Accelerated vascular disease seen in chronic renal failure, which accounts for significant morbidity and mortality, is also due to elevated homocysteine levels. In a double-blind placebo-controlled

randomized cross-over trial, researchers found that oral folic acid (5 mg/m^2) enhances serum folic acid (from 11.7 ± 4.25 to 635 ± 519 μg/L, $P = 0.001$) and red cell folic acid levels (from 364 ± 195 to 2891 ± 2623 μg/L, $P < 0.001$), decreases total homocysteine levels (from 10.28 ± 4.16 to 8.62 ± 2.32 μM/L, $P = 0.03$), improves flow-mediated dilatation (an endothelial-dependent dilatation), and prolongs the lag time for low-density lipoprotein oxidation.[24] Recent studies have suggested that folic acid improves endothelial function via mechanisms largely independent of decreasing homocysteine. Doshi et al.[25] administered oral folic acid (5 mg/d) and found that plasma folic acid can increase markedly by 1 h (200 versus 25.8 nM/L; $P < 0.001$), flow-mediated dilatation is improved 2 h (83 versus 51 μm; $P < 0.001$), but total and free homocysteine levels did not differ significantly at 4 h (9.56 versus 9.79 μM/L; *P*, not significant) and 3 h, although free homocysteine was slightly reduced at 4 h (1.55 versus 1.78 μM/L; $P = 0.02$). Flow-mediated dilatation improvement did not correlate with reduction in free or total homocysteine levels, suggesting that folic acid improves endothelial function in patients with CHD by a mechanism that is largely independent of plasma homocysteine concentrations.

Malondialdehyde and total plasma antioxidant capacity, markers of oxidative stress, remained unchanged after folic acid supplementation.[26] Conversely, 5-methyltetrahydrofolic acid (5-MTHF), when given intra-arterially, not only improved flow-mediated dilatation without altering plasma homocysteine concentrations but also abolished homocysteine-induced increase in intracellular superoxide generation. Folic acid and H_4B also abolished homocysteine-induced intracellular superoxide production in culture.[26] This suggests that folic acid, 5-MTHF, and H_4B (a cofactor essential of NO synthase that augments NO synthesis) restore endothelial function by suppressing superoxide production and enhancing NO generation or increasing its (NO) half-life. This is supported by the observation that 5-MTHF had no direct effect on in vitro NO production by eNO synthase (eNOS) but induced a dose-dependent reduction in eNOS- and xanthine oxidase–induced $O_2^{-\bullet}$ generation and reversed impaired endothelium-dependent vasodilation in patients with familial hypercholesterolemia.[27] The mechanism of this effect was attributed to the ability of 5-MTHF to reduce $O_2^{-\bullet}$ and enhance NO production.[28] 5-MTHF showed direct effects on the enzymatic activity of NO synthase in recombinant eNOS and cultured endothelial cells. It is also interesting to note that oral folic acid supplementation (10 mg/d) to healthy human volunteers not only restored NO synthesis to normal but also prevented nitrate tolerance to continuous treatment with nitroglycerin, possibly by restoring or stimulating endogenous regeneration of H_4B.[29]

H_4B, SUPEROXIDE ANION, AND NO

NO formation is critically dependent on the availability of the cofactor H_4B, which stimulates the conversion of L-arginine to L-citrulline and NO by NOS.[30] H_4B acts as a cofactor by providing electrons and in the process becomes oxidized to inactive quinonoid dihydrobiopterin.[31] H_4B restores impaired NO synthesis in hypercholesterolemia,[27,32] suggesting that H_4B can be used to restore the antiatherogenic potential of endothelial cells. This indicates that H_4B has a role as a therapy to increase NO synthesis to prevent atherosclerosis and other conditions in which increased production of NO is needed. However, H_4B is active only in its unstable and reduced form and thus not suitable for oral supplementation. Folic acid stimulates endogenous H_4B regeneration from quinonoid dihydrobiopterin.[33,34] This is one mechanism by which folic acid can restore NO formation and help in the prevention of atherosclerosis and CHD.

Insufficiency of H_4B leads to uncoupling of the L-arginine–NO pathway that can result in the increased formation of oxygen free radicals including $O_2^{-\bullet}$ by NOS and reduced NO production in vitro.[35–37] Insulin stimulates H_4B synthesis through the activation of guanosine triphosphate cyclohydrolase I, the rate-limiting enzyme in the de novo synthesis of H_4B in the aortic endothelium. In insulin-resistant state H_4B synthesis is decreased.[38] Thus, reduced NO production, due to decreased availability of H_4B, may be responsible for vascular abnormalities seen in the insulin-resistant states and type 2 diabetes mellitus.[38] Excess $O_2^{-\bullet}$ generation that occurs as a result of H_4B deficiency reacts with NO and inactivates it (NO) by limiting its (eNO) biological activity.[39] $O_2^{-\bullet}$ leads to the formation of hydroxyl radicals, which are toxic to endothelial cells and this in turn limits further generation of NO.[40] In rats Shinozaki et al.[41] showed that oral supplementation of H_4B (10 $mg \cdot kg^{-1} \cdot d^{-1}$) for 8 wk significantly increased H_4B content in the aorta, plasma, and erythrocytes in the treated compared with control rats, reduced endothelial $O_2^{-\bullet}$ generation and lipid peroxide content in the thoracic aorta and heart, restored endothelium-dependent vasodilation, and decreased sucrose-induced increase in blood pressure via the activation of eNOS. Treatment of fructose-fed rats (which develop insulin resistance and hypertension) with H_4B prevented the development of insulin resistance and inhibited the increased binding activity of nuclear factor-κB and activating protein-1 obtained from aorta and heart tissues, suggesting that H_4B is critical for the regulation of NOS activity.

INSULIN, H_4B, HYPERGLYCEMIA, STEROIDS, OXIDANT STRESS, AND NO

Depletion of H_4B and reduction in the H_4B/7,8-H_2B ratio may be critical for the regulation of NO and $O_2^{-\bullet}$ by endothelial cells.[37,38] Because supplementation of H_4B significantly increased the vascular content of H_4B, enhanced NO generation, and decreased $O_2^{-\bullet}$ production in the fructose-fed rats (which develop insulin resistance) and because insulin-resistant states induced a decrement in eNOS activity, it is reasonable to associate impaired H_4B synthesis in the insulin-resistant state with a fall in eNO synthesis.[41] Although the exact mechanism by which biopterin metabolism is altered in insulin-resistant states is not clear, insulin resistance per se and increased oxidative stress may contribute to impaired production of H_4B. Insulin stimulates H_4B synthesis via the activation of guanosine triphosphate cyclohydrolase I and dihydropteridine reductase,[42] and this action of insulin is impaired in the insulin-resistant state. In contrast, the biosynthesis of H_4B depends on a normal cellular redox state, and enhanced generation of free radicals impairs the endothelial recycling of H_4B.[43] Insulin-resistant states including obesity, hypertension, type 2 diabetes mellitus, and hyperglycemia are associated with increased vascular $O_2^{-\bullet}$ production[39,44–49] and 7,8-H_2B levels, which can result in enhanced oxidation of H_4B.[41] Decreased intracellular content of H_4B contributes to decreased formation of eNO. Thus, H_4B is a critical factor in maintaining endothelium-dependent vasodilation by reducing oxidative stress and maintaining synthesis of physiologic amounts of eNO. Further support to the role of H_4B in insulin resistance comes from the observation that, in non-diabetic, normotensive, and non-obese subjects, acetylcholine-induced vasodilation was positively correlated with insulin sensitivity, H_4B/7,8-H_2B ratio, and dihydropteridine reductase activity and inversely to increases in coronary lipid peroxide production. In addition, the H_4B/7,8-H_2B ratio was inversely correlated with dihydropteridine reductase activity and insulin sensitivity.[50] This suggests that abnormal pteridine metabolism is closely linked to endothelial dysfunction in insulin resistance.

Kanaya et al.[51] demonstrated in a canine model with stenosed and endothelium-injured coronary arteries mimicking acute coronary syndromes in humans that intraplatelet H_4B and cyclic guanosine monophosphate levels were decreased, intraplatelet nitrotyrosine production was increased, and ex vivo platelet aggregation and platelet P-selectin expression were augmented. These

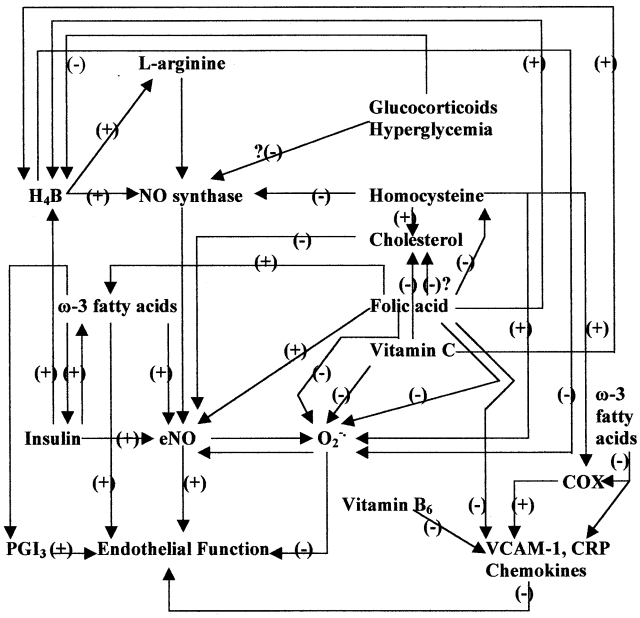

FIG. 2. Scheme showing the relation between homocysteine, folic acid, NO metabolism, H_4B, superoxide anion, and endothelial function. (+), increase in synthesis, action, or positive influence; (−), decrease or inhibition of synthesis, action, or negative influence; ?, relation not established; COX, cyclo-oxygenase; CRP, C-reactive protein; eNO, endothelial nitric oxide; H_4B, tetrahydrobiopterin; NO, nitric oxide; PGI_3, prostaglandin I_3; VCAM-1, vascular cell adhesion molecule-1.

abnormalities reverted to near normal after intravenous administration of H_4B (10 or 30 mg/kg), which was completely blocked by N(G)-monomethyl-L-arginine, an inhibitor of NO synthesis. Thus, intraplatelet H_4B plays a critical role in platelet function (especially thrombus formation) by modulating platelet-derived NO and $O_2^{-\bullet}$ production by platelet NOS. This is supported by the observation that H_4B improved endothelial dysfunction in coronary microcirculation in patients without epicardial CHD,[52] enhanced myocardial blood flow in healthy human volunteers,[53] and improved endothelial function in patients with CHD[54] by increasing NO availability. H_4B also improved endothelium-dependent vasodilation in nitroglycerin-tolerant rats[55] and in patients with type 2 diabetes mellitus[56] by increasing NO synthesis. In streptozotocin-induced diabetic rats, significantly reduced responses of the ocular vasculature to acetylcholine reverted to normal after administration of H_4B, indicating that a decreased level of H_4B is responsible for ocular vascular dysfunction.[57] High glucose and glucocorticoids decreased H_4B stability and synthesis, respectively, which led to inhibition of NO synthesis.[58,59] H_4B instability induced by high glucose was restored by ascorbic acid, whereas glucocorticoids-induced reduction in H_4B levels was due to inhibition of expression of guanosine triphosphate cyclohydrolase, the rate-limiting enzyme in the synthesis of H_4B. In both instances, H_4B restored endothelial function, suggesting that H_4B improves endothelial function in diabetes mellitus[60] and steroid-induced hypertension and prevents atherosclerosis.[61]

INTERACTIONS BETWEEN FOLATE/FOLIC ACID, H_4B, INSULIN, VITAMIN C, SUPEROXIDE ANION, AND eNO AND THEIR ROLES IN VASCULAR DISEASES

It is evident from the preceding discussion that folate/folic acid, H_4B, insulin, and $O_2^{-\bullet}$ interact with each other and significantly influence NO generation, stability, and action (Fig. 2). Folate/folic acid, H_4B, and insulin suppress $O_2^{-\bullet}$ production and thus prolong the half-life of NO.[11,62–64] This preserves endothelium-dependent vasodilation. Folate/folic acid, H_4B, and insulin possess anti-inflammatory actions by suppressing $O_2^{-\bullet}$ generation and enhancing NO production.[65] The ability of folate/folic acid and H_4B to interact with each other and enhance NO generation suggests that they could be useful in the prevention and treatment of insulin resistance, hypertension, CHD, and atherosclerosis. Folic acid and H_4B restore and attenuate cholesterol-induced endothelial dysfunction[27] and coronary hyperreactivity to endothelin,[66] respectively. Because folic acid restores the tissue stores of H_4B, folic acid and H_4B should be given together to obtain their maximum benefit. H_4B and stimulation of H_4B synthesis stimulated endothelial cell proliferation. N(G)-mono-methyl-L-arginine, a NO synthase inhibitor, attenuated H_4B-induced endothelial cell proliferation.[67] This suggests that H_4B levels regulate proliferation of normal endothelial cells and that its deficiency impairs NO-dependent proliferation of endothelial cells. Healthy endothelial cells are necessary for adequate production of NO and prostacyclin, which are potent platelet anti-aggregators and vasodilators, to prevent atherosclerosis and CHD. Folate/folic acid and H_4B are essential for NO synthesis and endothelial cell proliferation, which underscores their importance in the prevention and treatment of atherosclerosis per se and clinical conditions that accelerate atherosclerotic processes such as hypertension, hyperlipidemias, diabetes mellitus, and insulin resistance. In this context, it is interesting to note that mice lacking apolipoprotein E and low-density lipoprotein receptor genes, which develop atherosclerosis and endothelial dysfunction, showed pronounced enhancement of acetylcholine-induced relaxations when their thoracic aorta rings were incubated with L-arginine, the precursor of NO, and H_4B together but no or insignificant relaxations to L-arginine or H_4B alone.[68] One mechanism by which H_4B augments NO generation is by enhancing arginine transport in cardiac myocytes.[69] This suggests that, in the event plasma levels of L-arginine are low, H_4B may not be able to enhance eNO synthesis. Therefore, it is important to provide L-arginine, folic acid, or 5-MTHF, the active form of folic acid, H_4B, and vitamin C together in adequate amounts and in the right proportion to stimulate eNO synthesis. The importance of vitamin C (ascorbic acid) lies in the fact that it enhances eNOS activity by increasing intracellular levels[70,71] and by chemical stabilization of H_4B.[72] Vitamin C also enhances the release of NO[73] and thus suppresses the formation of increased amounts of total *S*-nitroso thiols and *S*-nitroso albumin.

H_4B AND NEUROTRANSMITTERS

H_4B is an essential cofactor not only for NO synthesis but also for tyrosine hydroxylase, tryptophan hydroxylase, and phenylalanine hydroxylase, which synthesize neurotransmitters, catecholamines, and serotonin, respectively. The brains of neonatal mutant knockout mice homozygous for 6-pyruvoyltetrahydropterin synthase (which catalyzes the second step of H_4B synthesis) showed extremely low levels of biopterin, catecholamines, and serotonin.[74] The number of tyrosine hydroxylase molecules was highly dependent on the intracellular concentrations of H_4B at nerve terminals, indicating that modulation of the H_4B content could be one novel regulatory mechanism of catecholamine and serotonin content in the brain. This may have relevance to various neurologic and neuropsychiatric conditions, which is supported by the observation that H_4B scavenges $O_2^{-\bullet}$, protects dopaminergic neurons from oxidative stresses generated by dopamine and its metabolites, and thus prevents Parkinson's disease.[75]

CONCLUSION AND CLINICAL IMPLICATIONS

What are the clinical implications of these interactions between folate/folic acid, H_4B, vitamin C, and insulin (which enhances

eNO synthesis)?[62] It is clear that these factors, molecules, or chemicals when present or given in adequate amounts augment NO synthesis and inhibit or prevent platelet aggregation, atherosclerosis, thrombosis, CHD, peripheral vascular disease, insulin resistance, and vascular abnormalities seen in diabetes mellitus, hypertension, and metabolic syndrome X and protect against the development of Parkinson' disease and other degenerative conditions of the brain. In chronic heart failure, markedly decreased NO availability due to impaired eNOS activity and enhanced $O_2^{-\bullet}$ production has been demonstrated,[76] suggesting that folic acid, H_4B, vitamin C, and insulin are useful. Impaired vasodilatation during alcohol consumption is due to a deficiency or alteration in the use of H_4B,[77] an event that can be corrected by supplementation of H_4B. In spontaneously hypertensive rats, H_4B supplementation suppressed the development of hypertension,[49] but it remains to be seen whether prophylactic use of L-arginine, folic acid, vitamin C, or H_4B can prevent the development of hypertension in those, at least, who are at high risk. Similarly, it is not known whether prophylactic use of these nutrients or agents can prevent insulin-resistance syndrome in humans. In this context, it is exciting to note that folic acid increases concentration of ω-3 polyunsaturated fatty acids (PUFAs) and decreases vitamin K–dependent coagulation factors, which could reduce the risk of thrombosis.[78–80] My colleagues and I showed that pretreatment with ω-3 fatty acids can prevent chemically induced diabetes mellitus in experimental animals,[81,82] attenuate insulin resistance,[83] and abrogate development of hypertension when given during the perinatal period.[84,85]

In homogenates of first-trimester or full-term placentae, H_4B stimulated NOS activity up to 2.5-fold in a concentration-dependent manner. Surprisingly, tissue concentrations of H_4B showed a marked decrease in term relative to first-trimester placentae, suggesting that cellular concentrations of H_4B may play a significant role in the regulation of NOS activity in late pregnancy. Placental homogenates showed two distinct types of responses to H_4B: in most instances, physiologic concentrations of H_4B elicited no increase in basal NOS activity; in only 30% of cases, H_4B stimulated NOS to levels similar to that of normal placentae. There were no significant differences in the clinical presentation between groups, and the H_4B concentrations in preeclamptic placentae were comparable with those of normal, control placentae.[86] This suggests that H_4B controls NOS activity in the human placenta and that a defect in this interaction between H_4B and NOS activity may have a significant role in the pathogenesis of preeclampsia. It is not known whether supplementation of H_4B and folic acid with vitamin C might abrogate the development of preeclampsia in high-risk pregnancy similar to the suppressive role of H_4B against the development of hypertension in spontaneously hypertensive rats.[49] Such a study would be interesting.

It is important to know that the beneficial action of H_4B on NO synthesis is not without controversy. For instance, exogenous H_4B impaired the action of eNO in the presence of platelets, which was inhibited by SOD, indicating a role for $O_2^{-\bullet}$. H_4B also blocked the anti-aggregatory effect of sodium nitroprusside, an NO donor, which was inhibited by a specific inhibitor of nicotinamide adenine dinucleotide phosphate oxidase,[87] indicating that H_4B stimulated $O_2^{-\bullet}$ generation from platelets (in the presence of nicotinamide adenine dinucleotide phosphate oxidase) that in turn inhibited the anti-aggregatory action of NO. In view of this finding, SOD activity in the local environment becomes critical in the relation between H_4B and eNO activity. In instances of low tissue concentrations of SOD, H_4B may actually do more harm than good, and this view is supported by the observation that H_4B played a redox role, albeit transiently, during the formation of NO.[88] Further, H_4B was cytotoxic to catecholamine cells but not to non-catecholamine cells in vitro, which was not dependent on the formation of dopamine or NO and was inhibited by catalase, SOD, peroxidase, and thiol agents. This indicated a role for reactive oxygen species. The H_4B cytotoxicity was initiated extracellularly, because enhancement of intracellular H_4B did not result in cell death.[89] H_4B is released spontaneously from the cells of its synthesis, which could represent one mechanism of damage to dopaminergic terminals and neurons and may result in Parkinson's disease. Abnormally low levels of H_4B may result in the production of reactive oxygen species, leading to the formation of peroxy nitrite, which oxidizes H_4B to quinonoid dihydrobiopterin, which readily forms 7,8-dihydropterin, which is not a cofactor for NO synthesis.[90] Thus, low tissue levels of H_4B likely promote a cycle of its own destruction. Hence, it is essential to ensure the presence of adequate amounts H_4B not only to maintain synthesis of NO but also to prevent further destruction of cells and tissues.

Folate deficiency reduces phosphatidylethanolamine methylation and could thus alter membrane phospholipid organization and function.[91] This suggests that folate or folic acid interacts intimately with other dietary factors such as PUFAs, which have many biological functions,[92,93] and thus brings about some of its biological actions and clinical benefits. H_4B is an important regulator of the action of NO released on platelet 12-lipoxygenase and cyclo-oxygenase activities (the precursors of which are PUFAs). Insulin is important for glucose homeostasis, enhancement of PUFA metabolism,[62] and stimulation of H_4B synthesis by activation of phosphatidyl 3-kinase.[94] This indicates that there is a close interaction between folic acid, H_4B, NO, insulin, PUFA and eicosanoid metabolism (Fig. 2).

Despite the biological importance of folate/folic acid in several diseases, it is paradoxical that there is still no general consensus as to the appropriate amounts of folic acid needed to obtain its full benefits,[95–97] although the actual upper limit recommended for folic acid is 1 mg/d. The American Heart Association (AHA) has recommended that the emphasis should be placed on meeting current recommended daily allowances for folate and vitamins B6 and B12 by the intake of vegetables, fruits, legumes, meats, fish, and fortified grains and cereals.[98] The AHA also recommended that the following individuals take 0.4 mg of folic acid, 2 mg of vitamin B6, and 6 μg of vitamin B12: patients with personal or family histories of premature cardiovascular disease; those with malnutrition, malabsorption syndromes, hypothyroidism, renal failure, or systemic lupus erythematosus; those taking certain medications, e.g., nicotinic acid, theophylline, bile acid-binding resins, methotrexate, and L-DOPA; or those with recent nitrous oxide exposure. A recent study[99] recommended 5 mg/d as safe and effective, although the actual recommended upper limit for folic acid is 1 mg/d. In view of the interaction of folic acid with other nutrients and agents, I suggest that a combination of folic acid (1 to 5 mg/d), vitamin B12 (1000 μg/d), vitamin B6 (5 to10 mg/d), vitamin C (100 mg/d), L-arginine (500 mg twice a day), H_4B (1 to 2 $mg \cdot kg^{-1} \cdot d^{-1}$ orally), PUFAs (especially eicosapentaenoic acid, 120 mg/d, and docosahexaenoic acid, 180 mg/d), and other nutrients may be necessary to derive its complete biological benefits.

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