Influence of Folate on Arterial Permeability and Stiffness in the Absence or Presence of Hyperhomocysteinemia

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Objective—Elevated plasma total homocysteine (tHcy) is associated with risk for cardiovascular disease. A common cause of mild hyperhomocysteinemia (HHcy) is folate deficiency. We sought to determine whether folate deficiency per se increases arterial permeability (quantitative fluorescence microscopy) and stiffness (vessel elastigraph), and whether the effects of folate deficiency are more severe in the presence of mild HHcy.

Methods and Results—Heterozygous cystathionine β-synthase (CBS)-deficient mice (CBS$^{+/-}$) and their wild-type littermates (CBS$^{+/+}$) were fed chow containing either standard (Con) or relatively low amounts of folate (LF) for 18 ± 3 weeks. Liver folate (μg folate/g liver) and tHcy (μM), respectively, were 12 ± 1 and 8 ± 1 in CBS$^{+/+}$ Con mice (n=12), and 8 ± 1 and 8 ± 1 in CBS$^{+/+}$ LF animals (n=5). Carotid arterial permeability was $\approx38\%$ greater ($P<0.05$) in CBS$^{+/+}$ LF versus Con mice, but vascular stiffening was unaltered. Liver folate and tHcy, respectively, were 13 ± 1 and 11 ± 1 in CBS$^{+/-}$ Con mice (n=16), and 8 ± 1 and 16 ± 3 in CBS$^{+/-}$ LF animals (n=6). Carotid arterial dextran accumulation was $\approx31\%$ greater, and maximal strain in aortae was $\approx20\%$ lower (both $P<0.05$) in CBS$^{+/-}$ LF versus Con mice.

Conclusion—Taken together, low folate ($P<0.05$) combined with mild HHcy ($P<0.05$) in CBS$^{+/-}$ mice produced more arterial dysfunction compared with low folate alone (ie, CBS$^{+/+}$ mice). These findings may be particularly relevant to elderly individuals because tHcy and deficiencies of folate metabolism increase with age. (*Arterioscler Thromb Vasc Biol.* 2006;26:814-818.)

Key Words: mice ■ cystathionine β-synthase ■ carotid artery ■ aorta ■ cardiovascular risk factors

Elevation of plasma total homocysteine (tHcy) is associated with an increased risk of cardiovascular disease, but the underlying mechanisms are not well understood.[1] Worldwide, the most common cause of mild hyperhomocysteinemia (HHcy) is deficiency of folate.[2,3] Some[4–8] but not all[9,10] studies suggest that folate deficiency contributes to cardiovascular disease in a manner that is independent of its ability to elevate plasma tHcy.[11] We have reported that HHcy evoked by folate depletion increases arterial permeability and stiffness in rats.[12]

Homocysteine is metabolized via the transsulfuration and remethylation pathways (Figure 1).[13] Cystathionine β-synthase (CBS) is the rate-limiting enzyme for homocysteine metabolism via the transsulfuration pathway. Heterozygous CBS-deficient (CBS$^{+/-}$) mice are predisposed to elevated tHcy concentrations.[14] Folate is required for homocysteine metabolism via the remethylation pathway. When folate consumption and/or absorption are compromised, plasma tHcy becomes elevated.[15]

In the present study, we sought to determine whether folate deficiency per se increases arterial permeability and stiffness in mice, and whether the effects of folate deficiency are more severe in the presence of mild HHcy. CBS$^{+/-}$ mice and their wild-type littermates (ie, CBS$^{+/+}$ mice) were fed chow containing either standard or relatively low amounts of folate. Arterial permeability and stiffness were assessed because of their potential relevance to atherosclerotic cardiovascular disease. In this regard, greater endothelial cell layer permeability facilitates arterial lipoprotein accumulation and thus may contribute to lesion development and/or severity, and decreased arterial compliance increases afterload to an extent whereby myocardial oxygen demand is elevated inappropriately.[16,17] Comparing CBS$^{+/+}$ mice on these 2 diets allowed us to test the hypothesis that low folate per se influences arterial permeability and stiffness. By also studying CBS$^{+/-}$ mice that consumed standard or low-folate chow, we were able to test the hypothesis that arterial dysfunction produced by low folate is more severe in the presence of mild HHcy.

Materials and Methods

All protocols used in this study were approved by the Animal Use and Care Committee at the University of California, Davis and

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Figure 1. The remethylation and transsulfuration pathways of homocysteine metabolism. Homocysteine remethylation is impaired when dietary folate is reduced. Homocysteine transsulfuration is impaired when cystathionine beta synthase enzyme activity is deficient. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; MTHF, methyltetrahydrofolate; MS, methionine synthase; CBS, cystathionine beta synthase; CBS$^{-/-}$, heterozygous for CBS deficiency.

conformed to guidelines set by the American Physiological Society and Animal Welfare Act.

Experimental Animals and Diets

Heterozygous CBS-deficient mice (CBS$^{-/-}$)[14] and their wild-type (CBS$^{-/-}$) littermates were housed individually under controlled temperature (23°C) and light conditions (12:12-hour light:dark cycle). CBS$^{-/-}$ mice were crossbred to C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Me) for at least 8 generations. Genotyping for the targeted CBS allele was performed by polymerase chain reaction.[14]

At the time of weaning, CBS$^{-/-}$ mice were fed a commercially available (Harlan Teklad, Madison, Wis) amino acid-defined diet containing either 0.75 mg (CBS$^{-/-}$ Control, Con; n=12) or 0.15 mg (CBS$^{-/-}$ low folate, LF; n=5) folate per 100 g of chow. CBS$^{-/-}$ mice were divided into 2 groups and treated similarly, ie, CBS$^{-/-}$ Con (n=16) and CBS$^{-/-}$ LF (n=6). Because homocysteine transsulfuration is compromised in CBS$^{-/-}$ mice, mild HHcy was predicted when these animals consumed the LF diet. All mice were given the antibiotic succinylsulfathiazole (1%) to eradicate intestinal microflora that are capable of synthesizing folate endogenously.

General Procedures

After 18±3 weeks on the respective diets, fasted mice were anesthetized (0.05 mg/g sodium pentobarbital, intraperitoneal), the chest opened, and blood was collected from the right ventricle to measure plasma tHcy. tHcy was measured using high-performance liquid chromatography (HPLC) with fluorescence detection[12,18] and refers to the combination of free reduced homocysteine (≈1% of total), mixed disulfides (20% to 30% of total), and protein-bound homocysteine (70% to 80% of total).[19] Samples of liver (for folate analysis) and thoracic aorta (to measure arterial elasticity) were removed, snap-frozen in liquid nitrogen (LN$_2$), and stored at −80°C. The right and left common carotid arteries were excised and prepared immediately to assess vascular permeability. Liver folate was measured using a conventional microbiological assay[12,20] because it is more indicative of long-term folate status and is less susceptible to fluctuations in metabolism than serum folate.[12,21,22]

Measurement of Arterial Permeability

Carotid arteries were placed in a microscope viewing chamber containing Krebs Henseleit (KH) buffer (pH 7.4, 37°C). The proximal and distal ends of each artery were cannulated and perfused with

1% bovine serum albumin at 2 mL/min (37°C, pH 7.4) for 15 minutes using a peristaltic pump. During this equilibration period, both arteries were viewed under a fluorescence microscope connected to a photometer and video camera while fluorescence intensity was recorded on a computer and chart recorder. The assessment of vascular permeability using quantitative fluorescence microscopy involves 3 perfusion phases. In the first, the artery was perfused (2 mL/min, 100 mm Hg, 37°C, pH 7.4, 8 minutes) with clear, nonfluorescent KH buffer plus 1% bovine serum albumin to measure baseline fluorescence intensity. Second, 4400 MW dextran molecules (estimated Stokes diameter, 1.4 nm; 42 μg/mL in perfusate) labeled with tetramethylrhodamine isothiocyanate (TRITC) (0.0833 mg/mL TRITC-dextran in perfusate, 494 nm excitation maximum, 518 nm emission maximum) were perfused through the arterial lumen for 5 minutes and viewed/recorded through an inverted light microscope. Dextran was used as the reference molecule because this nonlipid particle does not bind specifically to the artery wall. During the second phase a rapid increase in intraluminal fluorescence intensity occurs as TRITC-labeled dextran fills the artery lumen. In the third phase, the artery was perfused for 8 minutes with nonfluorescent buffer to wash the TRITC-labeled dextran out of the lumen. The 3 phases are collectively termed a perfusion "run."

The washout phase (ie, phase 3) is analyzed as 2 distinct processes. The first rapid washout represents dextran exiting the vessel lumen, whereas the second slower washout represents dextran exiting the vessel wall. Arterial permeability is estimated by the amount of TRITC-labeled dextran that accumulates in the arterial wall (I$_f$ accumulation). Calculating I$_f$ accumulation involves finding the intersection of tangents drawn to approximate the rapid and slow washout phases. To determine I$_f$ accumulation rate, I$_f$ accumulation is divided by time of perfusion. Fluorescence values then are converted from millivolts/min to ng TRITC-dextran/cm^2 per min^{-1} by knowing the: (1) surface area of the vessel in the photometric window; (2) arterial lumen volume of the vessel in the photometric window; (3) fluorescence intensity at time 0 (I$_f$ 0) that occurs at the beginning of TRITC perfusion; and (4) concentration of dextran in the perfusate. I$_f$ accumulation rates were performed in triplicate for each vessel, and the values were averaged.[12,23–28]

Measurement of Vascular Stiffening

Vascular stiffening was estimated using a modified vessel myograph, termed an elastigraph.[12] After vessels were thawed overnight, 2 stainless steel rods were inserted in a parallel manner through the lumen of a 1-mm segment of thoracic aorta while the vessel was immersed in KH buffer. One rod was fixed to a force transducer while the other was attached to a motorized controller. The elastigraph allows the vessel to be stretched radially at a constant rate until breakage while vessel tension is recorded via a force transducer. In preparation for each stretch, aortic segments were preconditioned 3 times at ≈10% of their maximal load (maximal tension at the vessel breaking point). Stress (vessel tension development divided by vessel area; N/mm^2) versus strain [(vessel width at breakage−vessel width at start) divided by vessel width at start; %] curves were generated using three 1-mm aortic segments from each animal and the results were averaged. From these curves maximal stress (ie, stress at vessel breakage; N/mm^2) and maximal strain (ie, strain at vessel breakage; ultimate extensibility, %) were calculated. Because samples from each group were thawed and analyzed together, any nonspecific side effects of freezing should have influenced both groups in an equivalent manner. These methods have been used previously by our laboratory and others.[12,17,26,29,30]

Drugs and Solutions

Unless noted otherwise, all chemicals were purchased from Sigma Chemical Co (St. Louis, Mo). KH solution contained (in mmol/L) NaCl (116), KCl (5), CaCl$_2$ · H$_2$O (2.4), MgCl$_2$ (1.2), NH$_2$PO$_4$ (1.2), glucose (11) and bovine serum albumin (1%).

Statistical Analyses

Animal and vessel characteristics, dextran accumulation rate, maximal strain, and maximal stress were compared among CBS$^{-/-}$ Con,

CBS$^{+/+}$ LF, CBS$^{+/-}$ Con, and CBS$^{+/-}$ LF groups using a 1-way ANOVA and a Tukey post-hoc test. Results are presented as mean±standard error of the mean. Statistical significance was accepted when $P<0.05$.

Results

General Characteristics

Body weight (g) was similar between CBS$^{+/+}$ Con (24±1) and LF (23±1) mice, and CBS$^{+/-}$ Con (22±1) and LF (21±1) animals. Whereas liver folate (μg/g liver) was ≈33% lower in CBS$^{+/+}$ LF versus CBS$^{+/+}$ Con mice, tHcy (μM) was similar between groups (Figure 2A and 2B). These results allowed us to evaluate the independent contribution(s) from low folate to arterial permeability and stiffness in CBS$^{+/+}$ animals without HHcy.
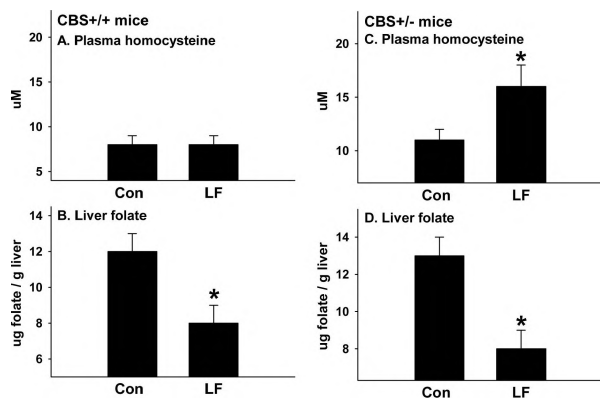
In CBS$^{+/-}$ LF mice, liver folate (μg/g liver) was ≈38% lower and tHcy (μM) was ≈1.5-fold higher compared with CBS$^{+/-}$ Con animals (Figure 2C and 2D). These results allowed us to determine whether the effects of low folate are more severe in the presence of mild HHcy.
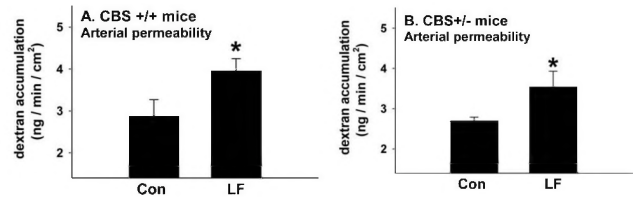
Arterial Permeability

Real-time measurements of dextran accumulation in the arterial wall were made using methods whereby flow rate, hydrostatic pressure, pH, temperature, and superfusate and perfusate compositions were controlled to simulate physiological conditions.[12,23–28] In CBS$^{+/+}$ mice, carotid arterial dextran accumulation was ≈38% greater in LF versus Con animals (Figure 3A). Likewise, arterial permeability was ≈31% greater in CBS$^{+/-}$ LF versus Con mice (Figure 3B). Thus, low folate is a major contributor to increasing arterial permeability and no further increase was observed in this variable when low folate and HHcy existed concomitantly.

Arterial Stiffening

The passive elastic properties of thoracic aortae were quantified using a vessel elastigraph modified to measure arterial elasticity.[12,17,30] This is a sensitive procedure compared with traditional compliance/distensibility methods.[17] Maximal strain was similar in thoracic aortae from CBS$^{+/+}$ LF and
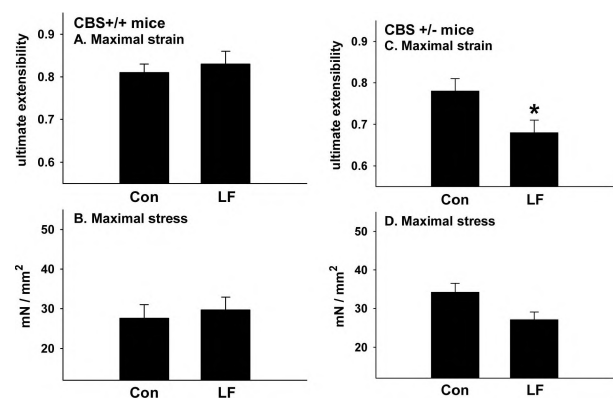

Figure 3. Arterial permeability was greater in CBS$^{+/+}$ (A) and CBS$^{+/-}$ mice (B) that consumed low-folate (LF) vs standard (Con) rodent chow. CBS$^{+/-}$ mice, mice that are heterozygous for cystathionine beta synthase deficiency; CBS$^{+/+}$ mice, wild-type littermates of CBS$^{+/-}$ mice. *$P<0.05$ HHcy vs Con. Values are mean±SEM.

CBS$^{+/+}$ Con mice (Figure 4A) but was ≈20% lower in thoracic aortae from CBS$^{+/-}$ LF versus CBS$^{+/-}$ Con mice (Figure 4C). Lower maximal strain indicates less distensible vessels, an outcome of increased vascular stiffening.[31] Maximal stress was similar among groups (Figure 4B and 4D). In comparison with the pathophysiological alterations on arterial permeability, increased vascular stiffening was dependent on both low-folate status and mild HHcy.

Discussion

Our findings support the hypotheses that low folate independently increases arterial permeability, and that both low folate and mild HHcy contribute to the severity of arterial stiffness. The clinical relevance of these findings is underscored by observations that tHcy increases with age,[32] and low to low–normal concentrations or deficiencies of folate resulting from reduced intake and/or decreased absorption are not uncommon in elderly individuals and/or populations that lack dietary fortification with folic acid.[33,34]

We manipulated the remethylation pathway of homocysteine metabolism in CBS$^{+/+}$ and CBS$^{+/-}$ mice via dietary means to generate experimental groups to test our hypotheses. Dietary folate restriction in CBS$^{+/+}$ mice produced significant reductions in liver folate but did not impair homocysteine remethylation to an extent that elevated plasma tHcy. Therefore, CBS$^{+/+}$ mice were used to test the hypothesis that low

Figure 2. tHcy was similar but liver folate was reduced in CBS$^{+/+}$ mice that consumed low-folate (LF) vs standard (Con) chow (A,B). tHcy was elevated and liver folate was reduced in CBS$^{+/-}$ LF vs Con mice (C,D). *$P<0.05$ LF vs Con. Values are mean±SEM. CBS$^{+/-}$ mice, mice heterozygous for cystathionine beta synthase deficiency; CBS$^{+/+}$ mice, wild-type littermates of CBS$^{+/-}$ mice.

Figure 4. Maximal strain (A) and maximal stress (B) were similar in CBS$^{+/+}$ mice that consumed low-folate (LF) and standard (Con) rodent chow. Maximal stress (D) was similar between groups but maximal strain (C) was lower in CBS$^{+/-}$ LF vs Con mice. *$P<0.05$ LF vs Con. Values are mean±SEM. CBS$^{+/-}$ mice, mice heterozygous for cystathionine beta synthase deficiency; CBS$^{+/+}$ mice, wild-type littermates of CBS$^{+/-}$ mice.

folate per se evokes arterial dysfunction. In CBS$^{+/-}$ mice, dietary folate restriction produced reductions in liver folate and elevations of plasma tHcy. This experimental approach allowed us to examine the combined influence of mild HHcy plus low folate on arterial permeability and stiffness.

Arterial Permeability: The Independent Influence of Low Folate

One of the initial steps in the development of atherosclerosis is endothelial dysfunction. This is frequently manifested as increased endothelial cell permeability. Multiple previous studies have shown that increased endothelial cell layer permeability is accompanied by the accumulation of low-density lipoprotein in the artery wall.[16,35] Increased permeability and lipoprotein accumulation could contribute to lesion development and/or severity.[16] Earlier we observed that HHcy-evoked by folate depletion increases arterial permeability in rats.[12] Because HHcy and low folate existed together in that study, their respective contributions to vascular dysfunction could not be discerned. Our current results support the previously untested hypothesis that low folate per se increases arterial permeability. In CBS$^{+/+}$ mice $\approx$33% reductions in liver folate were associated with 38% increases in carotid arterial permeability. Importantly, tHcy was similar between groups.

A strong rationale existed for performing this experiment. For instance, patients with low serum folate, but normal homocysteine, are known to have increased peripheral/coronary vascular disease.[4] Moreover, numerous investigations have shown that exogenous folic acid improves endothelial function in patients with cardiovascular disease in the presence[36–38] or absence[37–39] of homocysteine lowering. Together, these studies suggest a direct beneficial action of folic acid on vascular function. One proposed mechanism is that the active form of folic acid, 5-methyltetrahydrofolate, increases nitric oxide production, reduces O_2^- generation, and directly scavenges O_2^-.[40] As such, it follows that a critical reduction in folate may increase O_2^- generation and decrease nitric oxide bioavailability to an extent that elevates arterial permeability. In support of this model, we have found that when tissue folate was reduced by 50% in rats, liver lipid and protein oxidation were elevated, vascular O_2^- generation was increased, and vascular nitric oxide bioavailability was reduced.[5]

Arterial Stiffening: The Independent Influence of Low Folate

Arterial stiffness is a risk factor for cardiovascular disease[41] and is observed in patients with atherosclerosis, diabetes, and hypertension.[42] The effects of low folate per se on arterial stiffness have not been investigated, but several studies have examined whether folic acid supplementation improves this index of vascular function. In placebo-controlled, randomized, clinical trials, common carotid arterial stiffness was not altered in patients with end-stage renal disease[43] or in healthy individuals[44] who consumed folic acid for 1 to 2 years. In a third study, increased systemic arterial compliance was observed in folate-replete individuals who consumed 5 mg folic acid/day for 3 weeks compared with a matching placebo.[45] Collectively, the mixed results of these clinical trials do not

provide a clear picture regarding the effects of folic acid supplementation on arterial stiffness. Using CBS$^{+/+}$ mice, we examined the hypothesis that low-folate independently increases vascular stiffness in the absence of HHcy.[29] We found that when liver folate was reduced by $\approx$33%, no differences in arterial stiffening were observed in CBS$^{+/+}$ mice fed the LF diet. A limitation of this finding is that the severity of folate-lowering may not have been sufficient to influence arterial stiffness, despite a major effect on permeability. When we attempted to decrease liver folate to a greater degree in CBS$^{+/+}$ mice, remethylation was impaired to an extent that plasma tHcy was elevated, making it impossible to test the independent effect of low folate (data not shown).

Arterial Permeability and Stiffness: The Combined Influence of HHcy Plus LF

When 1.5-fold tHcy elevations were combined with $\approx$38% reductions of liver folate in CBS$^{+/-}$ LF mice, both arterial permeability and stiffness increased. These findings suggest that the vascular consequences of low folate are more severe when they coexist with mild HHcy. Increased arterial stiffening may result from the accumulation of advanced glycation end products (AGEs) that occurs during nonenzymatic glycation of elastin or collagen within the vascular wall.[29,31,46] During this process, oxidant stress produced by HHcy may stimulate the incorporation of glucose-derived crosslinks such as pentosidine between collagen fibers. Collagen crosslinking is one mechanism thought to be responsible for reduced vascular distensibility in diabetes and atherosclerosis, and could be operative in response to HHcy. In this regard, others have reported increased AGE receptor expression in mice with HHcy,[10] and we have shown that pentosidine is elevated 60-fold in arterial tissue from rats with HHcy that possess local and global indices of increased oxidant stress.[12]

In summary, we observed that reduction in liver folate resulted in increased arterial permeability, and that elevation of tHcy combined with reduction of liver folate produced both increased arterial permeability and stiffness. Greater arterial dysfunction in the setting of mild HHcy and low folate may be clinically relevant, especially for elderly individuals who may have deficiencies of folate resulting from a number of factors (eg, reduced intake, impaired absorption, interactions with medications).[33] These factors may be especially important in countries that do not recommend voluntary folate fortification of products by food manufacturers.[34,47]

Acknowledgments

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