Hyperhomocysteinemia increases arterial permeability and stiffness in mice


Hyperhomocysteinemia increases arterial permeability and stiffness in mice. Am J Physiol Regul Integr Comp Physiol 291: R1349–R1354, 2006. First published June 22, 2006; doi:10.1152/ajpregu.00335.2006.—We have reported that hyperhomocysteinemia (HHcy) evoked by folate depletion increases arterial permeability and stiffness in rats and that low folate without HHcy increases arterial permeability in mice. In this study, we hypothesized that HHcy independently increases arterial permeability and stiffness in mice. C57BL/6J mice that received rodent Chow and water (control [Con], n = 12) or water supplemented with 0.5% L-methionine (HHcy, n = 12) for 18 ± 3 wk had plasma homocysteine concentrations of 8 ± 1 and 41 ± 1 μM, respectively (P < 0.05), and similar liver folate (12 ± 2 μg folate/g liver). Carotid arterial permeability, assessed as dextran accumulation using quantitative fluorescence microscopy, was greater in HHcy (3.95 ± 0.4 ng·min⁻¹·cm⁻²) versus Con (2.87 ± 0.41 ng·min⁻¹·cm⁻²) mice (P < 0.05). Stress versus strain curves generated using an elastigraph indicated that 1) maximal stress (N/mm²), 2) physiological stiffness (low-strain Young’s modulus, mN/mm), and 3) maximal stiffness (high-strain Young’s modulus, N/mm) were higher (P < 0.05) in aortas from HHcy versus Con mice. Thus, chronic HHcy increases arterial permeability and stiffness. Carotid arterial permeability also was assessed in age-matched C57BL/6J mice before and after incubation with 1) xanthine (0.4 mg/ml)/xanthine oxidase (0.2 mg/ml; X/XO) to generate superoxide anion (O₂⁻) or 50 μM tetrahydrobiopterin (BH₄), which scavenged O₂⁻ in the presence of 2) vehicle, 3) 300 μM diethylamine- NONOate (DEANO; a nitric oxide donor), or 4) 10⁻³ M 4,5-dihydroxy-1,3-benzene disulfonic acid (tiron; a nonenzymatic intracellular O₂⁻ scavenger). Compared with preincubation values, X/XO and BH₄ homocysteine increased (P < 0.05) permeability by 66 ± 11% and 123 ± 8%, respectively. BH₄-homocysteine-induced increases in dextran accumulation were blunted (P < 0.05) by simultaneous incubation with DEANO or tiron. Thus, acute HHcy increases arterial permeability by generating O₂⁻ to an extent whereby nitric oxide bioavailability is reduced.

METHODS

All protocols used in this study were approved by Animal Use and Care Committees (University of California, Davis, CA, and University of Utah, Salt Lake City, UT) and conformed to guidelines set forth by the American Physiological Society and Animal Welfare Act.

General procedures. After an overnight fast, mice were anesthetized (0.05 mg/g pentobarbital sodium ip), the chest was opened, and blood was collected from the right ventricle to measure plasma total homocysteine (tHcy). Plasma tHcy was measured using HPLC with fluorescence detection (7, 37) and refers to the combination of free reduced homocysteine (~1% of total), mixed disulfides (20–30% of total), and protein-bound homocysteine (70–80% of total) (22). Next, samples of the liver (for folate analysis), thoracic aorta (to measure arterial elasticity), and abdominal aorta (for hydroxyproline quantification) were removed, snap frozen in liquid nitrogen, 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 (37, 40) because it is more indicative of long-term folate status and is less susceptible to fluctuations in metabolism than serum folate (4, 6, 37). Hydroxyproline was quantified to determine collagen function were impaired in rats (38), and carotid arterial permeability was greater in mice (39) that possessed low liver folate concentrations in the absence of HHcy.

In the present investigation, we tested the hypothesis that HHcy independently increases arterial permeability and stiffness in mice. Homocysteine is produced from dietary methionine through the intermediates S-adenosylmethionine and S-adenosylhomocysteine (Fig. 1). To produce HHcy without influencing folate status, L-methionine was added to the drinking water of mice. We chose to assess arterial permeability and stiffness because of their potential relevance to cardiovascular disease. In this regard, greater endothelial cell layer permeability facilitates arterial lipoprotein accumulation and contributes to lesion development and/or severity, and decreased arterial compliance increases afterload to an extent whereby myocardial oxygen demand may be elevated inappropriately (2, 30).

Cardiovascular risk; methionine; homocysteine; vascular mechanics

Hyperhomocysteinemia (HHcy) is a cardiovascular risk factor that may be caused by deficiencies of folate or B vitamins (e.g., cobalamin and pyridoxine) and/or by genetic defects in folate or methionine metabolism. We (37) have reported that HHcy evoked by folate depletion increases arterial permeability and stiffness in rats. HHcy, low folate, and/or their combination could have contributed to these responses. More recently, we (38, 39) found that a low folate status produces arterial dysfunction in a manner that is independent of its ability to elevate homocysteine. Specifically, conductance and resistance vessel

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content using a mini-Woessner microtiter plate method (36). The concentration of hydroxyproline was normalized to vessel length (2, 37).

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% BSA at 2 ml/min (pH 7.4, 37°C) for 15 min 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 involved three perfusion phases. In the first phase, the artery was perfused (2 ml/min, 100 mmHg, 37°C, pH 7.4, 8 min) with clear, nonfluorescent KH buffer and 1% BSA to measure baseline fluorescence intensity. Second, 4,400-molecular-mass dextran molecules (estimated Stokes diameter, 1.4 nm; 42 µg/ml in perfusate) labeled with tetramethylrhodamine isothiocyanate (TRITC; 0.0833 mg/ml) were perfused through the arterial lumen. The three phases were collectively termed as a perfusion “run.”

The washout phase (i.e., phase 3) was analyzed as two distinct processes. The first, rapid washout represents dextran exiting the vessel lumens, whereas the second, slower washout represents dextran exiting the arterial wall. Arterial permeability was estimated by the amount of TRITC-labeled dextran that accumulated in the arterial wall (I₆ accumulation). Calculating I₆ accumulation involved finding the intersection of tangents drawn to approximate the rapid and slow washout phases. To determine I₆ accumulation rate, I₆ accumulation was divided by the time of perfusion. Fluorescence values were then converted from millivolts per minute to nanograms of TRITC-dextran volume of the vessel in the photometric window; 2) arterial lumen volume of the vessel in the photometric window; 3) I₆ at time 0, which occurred at the beginning of the TRITC perfusion; and 4) concentration of dextran in the perfusate. I₆ accumulation rates were performed in triplicate for each vessel, and the values were averaged (5, 24–26, 37, 39, 46, 47).

In addition to perfusion studies performed on mice after the dietary interventions, five acute experiments were performed using age-matched C57BL/6J mice. First, carotid arteries were prepared for quantitative fluorescence microscopy, three perfusion runs were performed, and the results were averaged. Next, vessels were incubated for either 120 min with 1) 50 µM DL-homocysteine (n = 10); 2) 50 µM DL-homocysteine plus the nitric oxide (NO) donor diethylamine-NONOate (DEANO; 300 µM, n = 7); 3) 50 µM DL-homocysteine plus the nonenzymatic intracellular superoxide anion (O₂⁻) scavenger 4,5-dihydroxy-1,3-benzene disulfonic acid (tiron; 10⁻³ M, n = 2); or 4) 50 µM DL-homocysteine plus vehicle/volume (n = 4) for 5 min with 5) xanthine (0.4 mg/ml)/xanthine oxidase (X/XO; 0.2 mg/ml, n = 7) to generate O₂⁻ (17). After the respective incubation period, three perfusion runs were performed, and the results were averaged.

Measurement of vascular stiffening. Vascular stiffening was estimated using a modified vessel myograph, termed an elastograph (37). After vessels were thawed overnight, two stainless steel rods were inserted in a parallel manner through the lumen of a 1-mm segment of the thoracic aorta while the vessel was immersed in KH buffer. One rod was fixed to a force transducer, whereas the other was attached to a motorized controller. The elastograph allowed the vessel to be stretched radially at a constant rate until breakage while vessel tension was recorded via a force transducer. In preparation for each stretch, aortic segments were preconditioned three times at ±10% of their maximal load (maximal tension at the vessel breaking point). Stress (vessel tension development/vessel area, N/mm²) versus strain ([vessel width at breakage – vessel width at start]/vessel width at start, %) curves were generated using three 1-mm aortic segments from each animal, and the results were averaged. From these curves, the following parameters were calculated: 1) maximal stress, which was the stress at vessel breakage (N/mm²); 2) maximal strain, which was the strain at vessel breakage (ultimate extensibility, %); 3) physiological stiffness, which was the slope of the linear region between 30% and 40% strain of a stress versus strain curve (low-strain Young’s modulus, mN/mm); and 4) maximal stiffness, which was the slope of the linear region at high load values of a stress versus strain curve (high-strain Young’s modulus, N/mm) (2, 34, 37, 41).

Drugs and solutions. Unless noted otherwise, all chemicals were purchased from Sigma Chemical (St. Louis, MO). KH solution contained (in mmol/l) 116 NaCl, 5 KCl, 2.4 CaCl₂, 1.2 MgCl₂, 1.2 NH₄H₂PO₄, and 11 glucose with 1% BSA.

Statistical analyses. Animal and vessel characteristics, dextran accumulation rate, and indexes of vascular stiffening were compared between Con and HHcy mice using an unpaired Student’s t-test. For acute studies of arterial permeability, dextran accumulation rates before and after each respective incubation period were compared using a paired t-test. Results are presented as means ± SE. Statistical significance was accepted at P < 0.05.

RESULTS

Data from Con mice have been published previously (39) but were collected and analyzed concurrently with HHcy animals. Body weights were similar between Con (24 ± 1 g) and HHcy (25 ± 1 g) mice. Plasma tHcy was elevated by approximately fivefold in HHcy versus Con animals, but liver folate was not different between groups (Fig. 2, A and B). These results allowed us to evaluate the contribution from HHcy to arterial permeability and stiffness in the absence of folate deficiency.

Carotid arteries from HHcy mice were more permeable than those from Con animals. In this regard, dextran accumulation was ~38% greater in HHcy versus Con mice (Fig. 3). This
with dl-homocysteine. Specifically, the simultaneous incubation of dl-homocysteine plus DEANO blunted increases in dextran accumulation (3.29 ± 0.3 ng·min⁻¹·cm⁻²) versus Con (2.39 ± 0.29 ng·min⁻¹·cm⁻²). Likewise, a concomitant incubation of dl-homocysteine + tiron attenuated increases in dextran accumulation (2.04 ± 0.44 ng·min⁻¹·cm⁻²) versus Con (1.56 ± 0.34 ng·min⁻¹·cm⁻²). Figure 5 shows the percent increase from preincubation values after an incubation with X/XO, dl-homocysteine, dl-homocysteine plus DEANO, or dl-homocysteine plus tiron.

Internal control experiments measuring the time and volume effects of the perfusion system demonstrated no changes in arterial permeability. For example, the average dextran accumulation from three perfusion runs before (2.48 ± 0.22 ng·min⁻¹·cm⁻²) and after (2.41 ± 0.14 ng·min⁻¹·cm⁻²) a 120-min incubation with a vehicle/volume varied by 3 ± 7%.

DISCUSSION

Findings from this study support the hypothesis that arterial permeability and stiffness increase in response to chronic elevations of tHcy, even in the absence of folate deficiency. Results from acute experiments suggest that HHcy exerts its deleterious effects on arterial permeability by generating \( \text{O}_2 \) to an extent whereby NO bioavailability is reduced.

Arterial permeability. Previously, we (37–39) observed that HHcy evoked by folate depletion produced vascular oxidant stress and increased arterial permeability. Subsequently, Kamath et al. (11) compared vascular permeability between heterozygous cystathionine \( \beta \)-synthase (Cbs)-deficient (Cbs\(^{-/-}\)) mice that consumed methionine-enriched, low-folate chow for 8 wk (tHcy \( \sim 98 \) \( \mu \)M) and wild-type (WT) mice fed standard chow (tHcy \( \sim 4 \) \( \mu \)M). The authors reported greater permeability in the brain but not peripheral (e.g., skin, liver, and spleen) vasculature from Cbs\(^{-/-}\) versus WT mice. Because HHcy and low folate coexisted in both studies (11, 37), the relative contribution from each to increasing arterial permeability cannot be discerned. Recently, however, we (39) showed that low folate increased carotid arterial permeability in the absence of HHcy.

To determine the independent contribution from HHcy to increasing arterial permeability, we supplemented the drinking water of mice with l-methionine. Arterial permeability was assessed using real-time measurements of dextran accumulation in the vessel wall via methods whereby flow rate, hydro-

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**Fig. 2.** Plasma homocysteine (A) was elevated in hyperhomocysteinemia (HHcy) versus control (Con) mice. *\( P < 0.05 \), HHcy vs. Con. Liver folate (B) was similar between the groups. Values are means ± SE.

**Fig. 3.** Arterial permeability was greater in HHcy versus Con mice. *\( P < 0.05 \), HHcy vs. Con. Values are means ± SE.
static pressure, pH, temperature, and superfusate and perfusate compositions were controlled to simulate physiological conditions (5, 24–26, 37, 46, 47). We found that arterial permeability increased in response to high physiological/pathophysiological concentrations of tHcy (19, 33), thus suggesting an independent effect of HHcy.

HHcy-induced increases in arterial permeability may involve oxidant stress produced by tumor necrosis factor (TNF-α). TNF is a proinflammatory cytokine (20) that elevates O$_2^-$ in endothelial cells (27, 48) and vascular smooth muscle cells (21). Previously, we (46) showed that acute exposure to TNF increased the permeability of isolated perfused vessels and that this effect was attenuated by the antioxidants estradiol or α-tocopherol. Recently, Ungvari et al. (43) reported greater TNF expression in arteries from rats that consumed methionine-supplemented drinking water to produce HHcy. In that study (43), TNF increased O$_2^-$ production in vessels from HHcy animals by upregulating nox1-based NAD(P)H oxidase in arterial smooth muscle.

Results from our acute experiments have also implicated O$_2^-$ as a potential mechanism for elevated arterial permeability in response to HHcy. First, dextran accumulation was $\sim 70\%$ greater in carotid arteries after the incubation with X/XO, a well-known system for O$_2^-$ generation (17, 46). Second, the incubation with 50 $\mu$M homocysteine, a dose similar to the concentration (i.e., 41 $\pm$ 5 $\mu$M) experienced chronically by HHcy mice, elevated dextran accumulation by $\sim 120\%$ versus Con values obtained from the same vessel. Third, when carotid arteries were incubated with homocysteine in the presence of a potent, nonenzymatic intracellular O$_2^-$ scavenger (e.g., tiron) or a NO donor (e.g., DEANO), increases in arterial permeability were attenuated compared with results obtained in response to homocysteine alone. These findings demonstrate that acute (i.e., 120 min) elevations of homocysteine to levels experienced chronically (i.e., $\sim 18$ wk) in HHcy mice are capable of generating O$_2^-$ to an extent that reduces NO bioavailability and increases arterial permeability. Although these are acute experiments, they do provide a rationale for future studies to test whether increased vascular permeability produced by chronic HHcy can be attenuated by a simultaneous administration of antioxidants or NO donors, or be exacerbated by reducing NO bioavailability.

Although the precise mechanism(s) whereby O$_2^-$ limits NO bioavailability was not examined in the present study, several possibilities exist. For example, HHcy-induced O$_2^-$ could react with NO to produce peroxynitrite in the vascular wall (42). Not only does this reaction inactivate NO, but the resultant increase in vascular peroxynitrite has multiple adverse effects. Of these,
peroxynitrite can cause 1) tyrosine nitration of the major mitochondrial isoform of SOD and 2) oxidation of tetrahydrobiopterin. The former compromises the antioxidant capacity of the cellular environment, and the latter reduces the activity of endothelial NO synthase (eNOS) and/or promotes eNOS uncoupling, which is a circumstance wherein the normal flow of electrons within the eNOS enzyme is diverted so that superoxide rather than NO is produced (3, 10). Any and/or all of these mechanisms could be operative in the present study.

Arterial stiffening. Arterial stiffness is a risk factor for cardiovascular disease (14) and is observed in patients with atherosclerosis, diabetes, and hypertension (12). tHcy has been found to correlate strongly (1, 29), marginally (35), or not at all (13, 44, 45, 49) with arterial stiffness in experiments that have used a variety of methods to evaluate stiffness and increase tHcy. We evaluated the passive elastic properties of thoracic aortas using a vessel elastograph modified to measure arterial elasticity (2, 37, 41). This is a sensitive procedure compared with traditional compliance/distensibility methods (2, 31, 34). Maximal vascular stress and maximal and physiological stiffness increased markedly in aortas from HHcy versus Con mice.

The increased collagen content we observed in aortas from HHcy versus Con mice could have contributed to our findings because collagen is ~1,000 times stiffer than elastin (9, 23). Indeed, Majors et al. (18) reported that homocysteine enhanced collagen synthesis in cultured aortic smooth muscle cells, and Neves et al. (29) showed greater collagen deposition and vascular stiffness in mesenteric arteries from mice with mild elevations (~8 μmol/l) versus normal concentrations (~4 μmol/l) of tHcy (29). Furthermore, Mujumdar et al. (23) demonstrated severe elastin breakdown and impaired distensibility in rat aortic rings that were incubated with 100 μM homocysteine for 14 days versus Con arterial segments (23). Increased arterial stiffening could also result from greater collagen deposition combined with the accumulation of advanced glycation end products (AGEs) that occurs during nonenzymatic glycation of elastin or collagen within the vascular wall (32, 34, 50). During this process, oxidant stress stimulates the incorporation of glucose-derived cross-links such as pentosidine between collagen fibers. Collagen cross-linking is one mechanism thought to be responsible for the reduced vascular distensibility in diabetes and atherosclerosis and could be operative in response to HHcy. In this regard, others (8) have reported increased AGE receptor expression in mice with HHcy, and we (37) have shown that pentosidine is elevated 60-fold in arterial tissue from rats with HHcy that possess local and global indexes of increased oxidant stress.

Elevated tHcy is associated with increased risk for cardiovascular disease, but the underlying mechanisms are not well understood (for reviews, see Refs. 15 and 16). In the present study, high physiological/pathophysiological concentrations of tHcy (19, 33) evoked by methionine supplementation increased arterial permeability and stiffness in mice with normal folate status. Because one of the initial steps in the development of atherosclerosis is the accumulation of low-density lipoprotein in the artery wall (28, 30), HHcy could increase permeability, facilitate lipoprotein accumulation, and contribute to the enhanced lesion development that has been reported to occur in vessels from HHcy mice (30). Furthermore, aortic stiffening produced by HHcy could play a role in elevating cardiac afterload to an extent whereby ventricular wall stress and myocardial oxygen demand are elevated inappropriately. Taken together, our studies indicate that HHcy might contribute to cardiovascular disease, at least in part, by increasing arterial permeability and stiffness.

GRANTS

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