

## Vascular dysfunction produced by hyperhomocysteinemia is more severe in the presence of low folate

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**Symons, J. David, John C. Rutledge, U. Simonsen, and Roshny A. Pattathu.** Vascular dysfunction produced by hyperhomocysteinemia is more severe in the presence of low folate. *Am J Physiol Heart Circ Physiol* 290: H181–H191, 2006. First published September 2, 2005; doi:10.1152/ajpheart.00765.2005.—Earlier we reported that dietary folate depletion causes hyperhomocysteinemia (HHcy) and arterial dysfunction in rats (Symons JD, Mullick AE, Ensuna JL, Ma AA, and Rutledge JC. *Arterioscler Thromb Vasc Biol* 22: 772–780, 2002). Both HHcy and low folate (LF) are risk factors for cardiovascular disease. Therefore, the dysfunction we observed could have resulted from HHcy, LF, and/or their combination (HHcy + LF). We tested the hypothesis that HHcy-induced vascular dysfunction is more severe in the presence of LF. Four groups of rats consumed diets for ~10 wk that produced plasma homocysteine ( $\mu\text{M}$ ) and liver folate ( $\mu\text{g}$  folate/g liver) concentrations, respectively, of  $7 \pm 1$  and  $15 \pm 1$  (Control; Con;  $n = 16$ ),  $17 \pm 2$  and  $15 \pm 2$  (HHcy;  $n = 17$ ),  $10 \pm 1$  and  $8 \pm 1$  (LF;  $n = 14$ ), and  $21 \pm 2$  and  $8 \pm 1$  (HHcy + LF;  $n = 18$ ). We observed that maximal ACh-evoked vasorelaxation was greatest in aortas and mesenteric arteries from Con rats vs. all groups. While the extent of dysfunction was similar between LF and HHcy animals, it was less severe compared with arteries from HHcy + LF rats. Maximal ACh-evoked vasorelaxation in coronary arteries was not different between Con and LF rats, but both were greater than HHcy + LF animals. In segments of aortas, 1) ACh-evoked vasorelaxation was similar among groups after incubation with the nonenzymatic intracellular  $\text{O}_2^-$  scavenger Tiron, 2) vascular  $\text{O}_2^-$  estimated using dihydroethidium staining was greatest in HHcy + LF vs. all groups, and 3) tension development in response to nitric oxide (NO) synthase inhibition was greatest in Con vs. all other groups. We conclude that HHcy + LF evokes greater dysfunction than either HHcy alone (aortas, mesentery) or LF alone (aortas, mesentery, coronary), likely by producing more  $\text{O}_2^-$  within the vasculature and thereby reducing NO bioavailability.

coronary function; mesenteric artery function; nitric oxide bioavailability; superoxide anion; endothelium; vascular smooth muscle

STRONG EVIDENCE INDICATES that hyperhomocysteinemia (HHcy) is an independent predictor of cardiovascular disease (2, 35, 37, 39, 67, 68). At least one of the mechanisms whereby HHcy exerts its deleterious effects is by impairing endothelial function. For example, HHcy-induced reductions in endothelial function have been observed in experimental animals and humans (4, 25, 31, 32, 62). However, other studies show no association between HHcy and impaired endothelium-dependent vasorelaxation (9, 18, 20, 42).

In an earlier investigation we reported that dietary folate depletion causes moderately severe HHcy ( $\sim 58 \mu\text{M}$ ) and resistance and conductance vessel dysfunction in rats (49).

Because folate is required for the remethylation of homocysteine to methionine, decreasing its availability experimentally is a reliable and reproducible method to produce HHcy. However, this procedure has limitations. In this regard, some (3, 21, 42) but not all studies (23, 59) indicate that low folate contributes to cardiovascular disease in a manner that is independent of its ability to elevate homocysteine. Therefore, the vascular dysfunction we observed earlier potentially could have resulted from an independent contribution from HHcy, low folate, and/or their combination.

Since the publication of our earlier study (49), Ungvari et al. (55) administered L-methionine to drinking water of rats and showed the resultant HHcy impairs flow-mediated coronary vasodilation by a superoxide anion-mediated reduction of nitric oxide (NO) bioavailability. These data suggest strongly that HHcy evokes endothelial dysfunction in an independent manner. The contribution from low folate to endothelial dysfunction is less clear. In this regard, although several reports indicate an association exists between low serum folate and impaired endothelial function (3, 21, 42, 61), the hypothesis that low folate independently causes vascular dysfunction has never been tested. In contrast, numerous investigations have shown exogenous folic acid improves endothelial dysfunction in patients with cardiovascular disease in the presence (5, 10, 54) and absence (10, 60) 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 (5-MTHF), increases NO production, reduces superoxide production, and directly scavenges superoxide anion (45). Therefore, a critical decrease in folate could impair NO bioavailability via decreased production and/or increased inactivation of this important vasoactive/antiatherosclerotic molecule. If both HHcy and low folate concentrations increase oxidant load and compromise NO bioavailability, it is reasonable to hypothesize that HHcy-induced vascular dysfunction is exacerbated in the setting of low circulating folate concentrations. This is a clinically relevant scenario, particularly in elderly individuals (34).

The purpose of the present study was to test the hypothesis that HHcy-induced vascular dysfunction is more severe in the presence of low folate. Specifically, we evaluated the extent to which HHcy, low folate, and their combination produce superoxide anion, decrease NO bioavailability, and impair vasorelaxation of resistance and conductance arteries.

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## METHODS

**Preliminary experiment to determine diets.** To test our hypotheses, it was necessary to establish a degree of methionine supplementation required to evoke clinically relevant HHcy. Furthermore, it was necessary to determine a dietary folate content that reduced liver folate to the greatest extent possible without elevating homocysteine. With the use of protocols approved by the Animal Use and Care Committee at the University of Utah, male weanling rats consumed one of six combinations of water supplemented with L-methionine (0%, 0.5%, 1.0%) and rodent chow containing different folate contents (10, 0.8, and 0.4 mg folate/kg feed;  $n = 8$  per group, 48 animals total). After 10 wk, total plasma homocysteine (tHcy) was measured using HPLC with fluorescence detection (16, 49). tHcy 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). Liver folate was measured (conventional microbiological assay) (49, 52) because it is more indicative of long-term folate status and is less susceptible to fluctuations in metabolism than serum folate (12, 15). Diets used to test our hypotheses were chosen on the basis of data obtained from these preliminary studies.

**Preliminary experiment to measure intraluminal NO concentration and vascular force in coronary arteries.** Directly measured NO is compromised in endothelial cells exposed to high concentrations of Hcy (64). Whereas we (49) and others (55) have used pharmacological probes to show that NO bioavailability is less in coronary arteries from HHcy vs. control rats, no studies have directly measured NO in coronary arteries. We hypothesized that intraluminal NO concentrations ([NO]) would be less in coronary arteries from HHcy vs. control rats. Male weanling rats (50–75 g,  $n = 20$ ) were purchased from Mollegaard Breeding Center, Skensved, Denmark. With the use of protocols approved by the Animal Use and Care Committee at the University of Aarhus, rats consumed standard rodent chow and drinking water that was ( $n = 10$ ) or was not ( $n = 10$ ) supplemented with 1% methionine. After 10 wk, animals were anesthetized using 2–5% isoflurane. The caudal artery was cannulated, and the catheter was tunneled subcutaneously to exit between the scapulae (44, 51). Anesthesia was discontinued, animals regained consciousness within 5 min, and 60–90 min later a blood sample was obtained to assess plasma homocysteine. Next, animals were anesthetized deeply, the chest was opened, the heart was removed and placed in iced normal physiological saline solution (NPSS, pH ~ 7.40), and segments of liver were immersed in liquid nitrogen to later assess folate.

The left coronary artery was isolated carefully from surrounding tissue and mounted on a wire myograph (41, 43). After a 30-min equilibration period at zero resting tension, 1) the vessel diameter that evokes the greatest tension development ( $L_{\max}$ ) was determined, 2) vasoconstriction to 125 mM KCl was quantified, and 3) a calibrated (see below) NO-sensitive microelectrode (ISONOP30, World Precision Instruments) was inserted into the myograph chamber through a hole drilled in one side that was sealed with high vacuum grease. Steps 1–3 each were separated by 60-min. The NO-sensitive microelectrode (length 0.5–2 mm) was placed into the artery lumen using a microscope and micromanipulator, while vessel force was monitored to ensure that contact with the endothelial surface was avoided. The NO electrode was connected to an amplifier (NO meter, World Precision Instruments), and the signal was registered on a recorder permitting simultaneous measures of NO and vascular tension. Three protocols, each separated by 45–60 min, were performed on each vessel. Vascular tension and [NO] were monitored in response to 1) the NO scavenger oxyhemoglobin (OxyHb, 10  $\mu$ M), 2) endothelium-dependent vasorelaxation evoked by ACh ( $10^{-7}$ – $10^{-5}$  M), and 3) endothelium-independent vasorelaxation produced by the NO donor S-nitroso-N-acetylpenicillamine (SNAP,  $10^{-8}$ – $10^{-6}$  M). Responses to ACh and SNAP were evaluated in vessels precontracted with  $\sim 10^{-7}$ – $10^{-6}$  M 9,11-dideoxy-9 $\alpha$ ,11 $\alpha$ -methanoepoxy-prosta-(5Z,13E)-dien-1-oic acid (U-46619).

Calibration of the NO electrode was performed in the myograph before and after each experiment by adding known NO concentrations (3–100 nM) prepared from deoxygenated (argon-bubbled) distilled water. Detailed explanation of the calibration procedure and experiments evaluating selectivity of the electrode are provided elsewhere (41).

**Experimental animals and diets.** All protocols used in the main study were approved by the Animal Use and Care Committee at the University of Utah and conformed to guidelines set by the American Physiological Society and Animal Welfare Act. Weanling male Sprague-Dawley rats (50–75 g) were housed individually under controlled temperature (23°C) and light conditions (12:12-h light-dark cycle) and were allowed standard rodent chow and water ad libitum for ~1 wk after arrival from the breeder (Charles River Laboratories, Wilmington, MA). Animals then were divided into four groups. Two groups received an amino acid defined diet containing 10 mg of folate/kg of chow (Harlan Teklad Diet TD99105). Of these, one group consumed standard drinking water (Con,  $n = 16$ ), while the other consumed water supplemented with 1% methionine (HHcy,  $n = 17$ ). Two additional groups received an amino acid defined diet containing 0.4 mg folate/kg of chow (Harlan Teklad Diet TD99331). Of these, one group consumed standard drinking water [low folate (LF),  $n = 14$ ], while the other consumed water supplemented with 1% methionine (HHcy + LF,  $n = 18$ ). The antibiotic succinylsulfathiazole (1%) was added to all diets to eradicate intestinal microflora capable of synthesizing folate endogenously. All rats consumed their respective diets for 10 wk.

**General procedures.** After a 12-h fast, animals were anesthetized using 2–5% isoflurane. The caudal artery was cannulated and the catheter was tunneled subcutaneously to exit between the scapulas (44, 51). Anesthesia was discontinued, animals regained consciousness within 5 min, and 60–90 min later systemic arterial pressure and heart rate were obtained over at least 20 cardiac cycles. Next, arterial blood samples were collected to measure glucose (Glucometer Elite), tHcy (HPLC with fluorescence detection) (16, 49), and hematocrit. After blood sampling, the animals were anesthetized deeply (3–5% isoflurane), the chest was opened, and the heart, aorta, mesenteric arteries, and liver were excised. Vascular reactivity was determined using coronary arteries, segments of thoracic aortas, and mesenteric arteries. Additional segments of aorta were used to detect vascular superoxide anion ( $O_2^-$ ), Cu-Zn + Mn superoxide dismutase activity (Cu-Zn + Mn SOD; kinetic spectrophotometric assay) (36, 49), and indexes of medial thickening. Segments of liver were used to quantify folate (conventional microbiological assay) (49, 52), protein carbonyls (spectrophotometric quantification of the dinitrophenylhydrazine adduct) (33, 48, 49), and thiobarbituric acid reactive substances (fluorescence detection) (13, 48, 49). Only measurement techniques that we have not published previously (i.e., vascular  $O_2^-$  detection, aortic medial thickening) are described in greater detail.

**Measurement of vascular reactivity.** Vessels were mounted on a wire-type myograph while immersed in a temperature-controlled, 8-ml tissue “bath” containing oxygenated (95%  $O_2$ -5%  $CO_2$ ) NPSS (pH ~ 7.40). After the arteries were mounted, the tissue bath was warmed gradually to 37°C over 30-min with vessels at 0-mg tension. During this time and throughout each experiment, the pH and temperature of all buffer solutions were checked at 30-min intervals, and contents of the tissue bath were exchanged at 15-min intervals. Experimental protocols specific to each vessel type are described.

**Aortas.** When the tissue bath reached 37°C, tension on aortas was increased manually over 60 min to 2 g. Final tensions did not differ among groups (i.e.,  $2,038 \pm 17$  mg). After 30 min, vessels were precontracted with norepinephrine (NE,  $10^{-7}$  M) and vasorelaxation responses to ACh ( $10^{-8}$ – $10^{-4}$  M; to determine endothelium-dependent vasorelaxation), ACh in the presence of 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron;  $10^{-3}$  M; to determine endothelium-dependent vasorelaxation in the absence of superoxide anion), and sodium nitroprusside (SNP,  $10^{-9}$ – $10^{-4}$  M; to determine endothelium-

independent vasorelaxation) were performed. In addition, vasocontractile responses to NE ( $10^{-8}$ – $10^{-4}$  M; to determine receptor-mediated vasoconstriction) and KCl (10–100 mM; to determine non-receptor-mediated vasoconstriction) were assessed. Vasocontractile responses to  $N^G$ -methyl-L-arginine (L-NMMA,  $10^{-3}$  M) were evaluated in NE-precontracted vessels to estimate basal NO production (29). Each experimental protocol was separated by at least 30 min.

Mechanisms responsible for impaired vasorelaxation were studied using segments of aortas because our past and present data from this vessel indicate that atropine abolishes ACh-evoked vasorelaxation, L-NMMA attenuates ACh-evoked vasorelaxation by  $\geq 90\%$ , removal of the endothelium abolishes ACh-evoked vasorelaxation, and ACh evoked vasorelaxation is repeatable when concentration-response curves are separated by  $\sim 30$  min (11, 46, 48–50). Furthermore, L-NMMA-resistant/NO-independent vasodilatory pathways exist in rat coronary and mesenteric arteries. Collectively, these findings indicate that in segments of aortas, ACh stimulates muscarinic receptors to evoke repeatable endothelium-dependent vasorelaxation that is mediated almost exclusively by NO.

**Coronary arteries.** Vessel tension was increased manually to 10 mg when the vessel chamber reached  $37^\circ\text{C}$ . Thirty minutes later, a series of internal circumference-active tension curves was constructed to determine the vessel diameter that evoked the greatest tension development ( $L_{\max}$ ) to 100 mM potassium chloride (KCl).  $L_{\max}$  tension ( $412 \pm 16$  mg) did not differ among groups. On vessels that were precontracted to  $73 \pm 7\%$  of maximal developed tension with endothelin-1 (ET-1;  $\sim 3 \times 10^{-8}$  M), dose-response curves to ACh ( $10^{-8}$ – $10^{-4}$  M) and SNP ( $10^{-9}$ – $10^{-4}$  M) were performed.

**Mesenteric arteries.** When the vessel chamber reached  $37^\circ\text{C}$ , tension was increased manually to 200 mg over 10 min. Thirty minutes later,  $L_{\max}$  was determined and did not differ among groups ( $750 \pm 36$  mg). On vessels that were precontracted with NE ( $10^{-7}$  M), vasorelaxation responses to ACh ( $10^{-8}$ – $10^{-4}$  M) and SNP ( $10^{-9}$ – $10^{-4}$  M) were performed. In addition, vasocontractile responses to NE ( $10^{-8}$ – $10^{-4}$  M), KCl (10–100 mM), and L-NMMA (in NE-precontracted vessels) were recorded. For all vessels studied, vasorelaxation is expressed as percent relaxation from precontraction tension, and vasoconstriction is presented as milligrams of developed tension (NE, KCl) or as percent increase from precontraction tension (L-NMMA). For all protocols, the appropriate time, volume, vehicle, and repeatability controls were performed. All tension data were recorded continuously by a computer through an analog-to-digital interface card (Biopac Systems, Santa Barbara, CA) that allowed for subsequent off-line quantitative analyses (11, 46, 48–50).

**Detection of vascular  $O_2^-$ .** Vascular  $O_2^-$  was detected using the oxidative fluorescent dye dihydroethidium (DHE; Molecular Probes, Eugene, OR) (7, 19). Fresh segments of aorta were frozen in optimum cutting temperature (OCT) compound immediately after excision. Eight 10- $\mu\text{m}$  frozen sections were cut and studied each day. Of these, four were treated with DHE (4  $\mu\text{mol/l}$  concentration; 200- $\mu\text{l}$  volume per section) and four were treated with PBS (200  $\mu\text{l}$  volume; vehicle/time control). All sections were incubated at  $37^\circ\text{C}$  in a light-protected, humidified chamber for 30 min and rinsed once with 400  $\mu\text{l}$  of PBS to remove unoxidized DHE.  $O_2^-$  production was estimated using confocal microscopy (Olympus Fluoview; Olympus America, Melville, NY). The settings for laser scan imaging included  $512 \times 512$  pixel resolution; argon/krypton laser power, 6%; objective,  $\times 60$  NA 1.2; ethidium bromide (EtBr) excitation, 488 nm; EtBr emission, 580- to 630-nm band-pass filter. Identical photomultiplier tube voltage (725 V) and gain (2.0) settings were used for all sections. Four fields of view (FOV,  $235 \mu\text{m} \times 235 \mu\text{m}$ ) from each vascular ring were imaged such that no regional overlap occurred. Maximum intensity  $z$ -projections were used to quantify relative fluorescence intensity (RFI) using the NIH IMAGEJ program (Bethesda, MD). Data are reported as RFI units from 0 (least intensity) to 255 (greatest intensity). This unit is arbitrarily defined by the digitizer gain, which was held constant for all samples.

**Vascular morphology.** Aortic segments were fixed in formalin (24 h) and stored in ethanol before paraffin embedding. Sections (4  $\mu\text{m}$ ) were mounted on slides and stained with hematoxylin and eosin. Tunica media thickness was measured from calibrated digital photographs using NIH Image 1.32 software (<http://rsb.info.nih.gov/ij/>).

**Drugs and solutions.** All chemicals were obtained from Sigma Chemical (St. Louis, MO) unless noted otherwise. NPSS contained (in mM) 125 NaCl, 4.7 KCl, 1.2  $\text{KH}_2\text{PO}_4$ , 1.2  $\text{MgSO}_4$ , 2.5  $\text{CaCl}_2$ , 18  $\text{NaHCO}_3$ , 0.026  $\text{Na}_2\text{EDTA}$ , and 11.2 glucose. ACh, SNP, L-NMMA, NE, and KCl were prepared daily from stock solutions by using distilled deionized water. DHE was reconstituted by using DMSO.

**Statistical analyses.** For preliminary studies, plasma and liver folate were analyzed among the various dietary combinations by using a one-way ANOVA. Tukey post hoc tests were performed to determine the location of the difference(s) if significance was obtained. Animal and vessel characteristics and oxyhemoglobin (OxyHb)-evoked vasocontractile responses and [NO] values were compared between Con and HHcy animals using an unpaired  $t$ -test. ACh and SNAP dose-response curves were analyzed using a two-way (time or drug dose vs. experimental group) repeated-measures ANOVA. If significance was attained, planned comparisons were made at each drug dose to determine the location of difference(s) among groups.

For the main study, animal and vessel characteristics, plasma and tissue markers, and L-NMMA-evoked vasocontractile responses were compared among groups using a one-way ANOVA. Vascular dose-response curves were analyzed using a two-way (time or drug dose vs. experimental group) repeated-measures ANOVA. If significance was attained, planned comparisons were made at each drug dose to determine the location of difference(s) among groups. For vascular studies, when the same protocol was performed on more than one vessel from a single animal, responses were averaged and counted as one observation. Results are presented as means  $\pm$  SE. Statistical significance was accepted at  $P < 0.05$ .

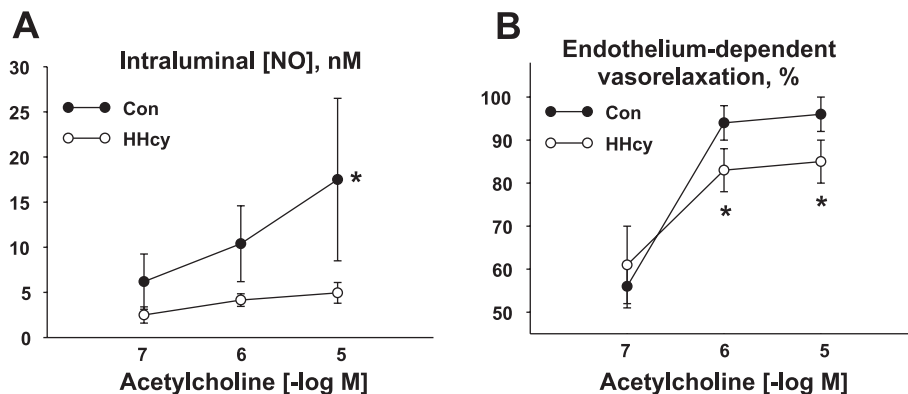
## RESULTS

**Preliminary experiment to determine diet.** One percent methionine in drinking water for 10 wk sufficiently elevated tHcy, and 0.4 mg folate/kg chow significantly lowered liver folate to an extent that did not elevate tHcy. The combination of 1% methionine in drinking water plus 0.4 mg folate/kg chow elevated tHcy to the same degree as 1% methionine alone and lowered folate to the same extent as 0.4 mg folate/kg chow alone. Data from these preliminary studies are not shown.

**Preliminary experiment to determine intraluminal [NO] and vascular force.** Body weight ( $405 \pm 8$  vs.  $427 \pm 9$  g) and liver folate ( $18 \pm 2$  vs.  $16 \pm 2$   $\mu\text{g}$  folate/g liver) were similar in HHcy and Con rats, respectively, but plasma tHcy was greater in HHcy ( $16 \pm 2$   $\mu\text{M}$ ) vs. Con ( $4 \pm 2$   $\mu\text{M}$ ) animals. Resting internal diameter ( $429 \pm 19$  vs.  $450 \pm 11$   $\mu\text{m}$ ),  $L_{\max}$  internal diameter ( $486 \pm 19$  vs.  $500 \pm 12$   $\mu\text{m}$ ), and coronary artery length ( $1.73 \pm 0.05$  vs.  $1.89 \pm 0.03$  mm) was similar in HHcy vs. Con animals, respectively.

Tension development from rest (mg) in response to 10  $\mu\text{M}$  OxyHb was less in coronary arteries from HHcy ( $42 \pm 19$ ) vs. Con rats ( $90 \pm 30$ ). Likewise, OxyHb-induced reductions from baseline in intraluminal [NO] (nM) were less in HHcy ( $6 \pm 3$ ) vs. Con rats ( $22 \pm 6$ ). These results suggest HHcy reduces basal intraluminal NO bioavailability. In vessels precontracted to the same degree ( $78 \pm 4\%$ ), ACh-evoked intraluminal [NO] and vasorelaxation responses were less in HHcy vs. Con rats (Fig. 1, A and B, respectively). These results indicate stimulated NO bioavailability is less in arteries from HHcy vs. Con rats. Original tracings are shown in Fig. 2, A and B. Finally, in

Fig. 1. Intraluminal nitric oxide concentrations ([NO]) (A) and vascular force (B) in coronary arteries from control (Con) rats and those with hyperhomocysteinemia (HHcy) in response to ACh. \* $P < 0.05$ , HHcy vs. Con. Values are means  $\pm$  SE.



vessels precontracted to the same degree ( $76 \pm 8\%$ ), intraluminal [NO] and vasorelaxation responses to SNAP were similar in HHcy and Con rats (Fig. 3, A and B, respectively). These findings indicate coronary vascular smooth muscle function is similar between groups.

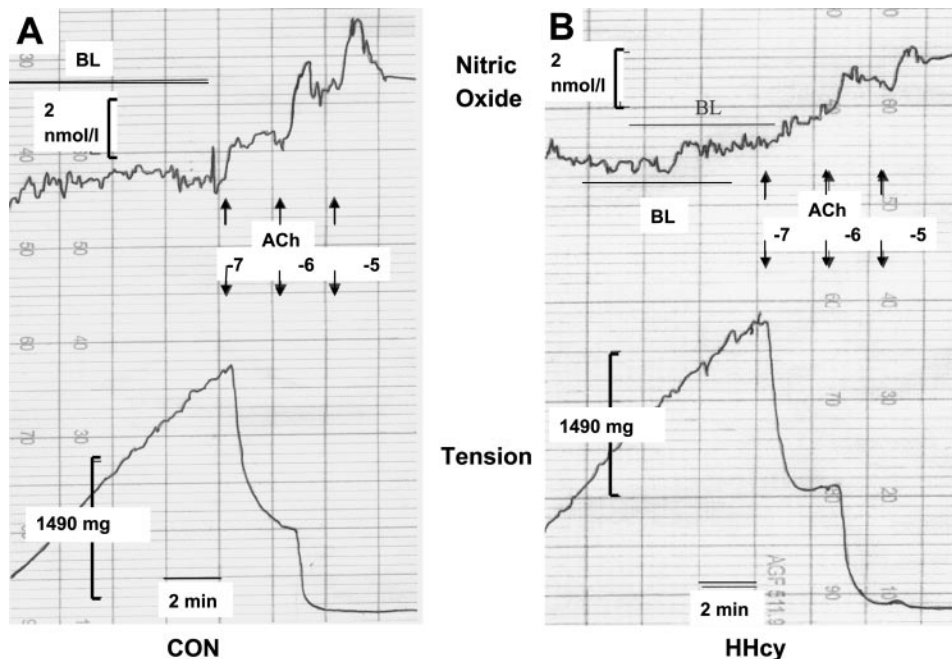
**General animal characteristics.** Animals appeared healthy and robust at the time of study, and their characteristics are shown in Table 1. Figure 4 indicates that 1) tHcy was elevated  $\sim 2.4$ -fold in HHcy vs. Con animals (Fig. 4A); 2) liver folate was reduced by  $\sim 50\%$ , but tHcy concentrations were similar, in LF vs. Con rats (Fig. 4B); and 3) tHcy was elevated to the same degree, and liver folate was reduced by  $\sim 50\%$ , in HHcy + LF vs. HHcy animals. These results allowed us to evaluate the independent contribution(s) to vascular dysfunction from HHcy and LF and to determine whether HHcy-induced vascular dysfunction is exacerbated in the presence of low folate.

**Vascular function.** Characteristics for all vessels are shown in Table 1. In aortas, maximal ACh-evoked vasorelaxation was greatest in Con rats ( $94 \pm 7\%$ ) vs. all other groups (Fig. 5A). While maximal vasorelaxation was not different between vessels from LF ( $81 \pm 3\%$ ) and HHcy ( $74 \pm 8\%$ ) animals, both

were greater vs. arteries from HHcy + LF rats ( $56 \pm 6\%$ ). For mesenteric arteries, maximal ACh-evoked vasorelaxation was greatest in Con rats ( $90 \pm 2\%$ ) vs. all other groups (Fig. 5B). While maximal vasorelaxation was not different between vessels from LF ( $62 \pm 10\%$ ) and HHcy ( $65 \pm 12\%$ ) animals, both were greater vs. arteries from HHcy + LF rats ( $43 \pm 7\%$ ). Maximal ACh-evoked vasorelaxation was similar in coronary arteries from Con ( $64 \pm 6\%$ ) and LF ( $53 \pm 8\%$ ) animals (Fig. 5C). Vasorelaxation was impaired in HHcy ( $43 \pm 6\%$ ) and HHcy + LF ( $35 \pm 7\%$ ) vs. Con rats. In general, endothelium-dependent vasorelaxation was impaired in aorta, mesenteric, and coronary arteries to the greatest degree by the combination of HHcy + LF. Endothelium-independent vasorelaxation produced by SNP was similar among groups for aortas, mesenteric, and coronary arteries (Fig. 6, A–C, respectively).

When precontraction to NE was stable in aortas, L-NMMA was administered, and the percent increase in tension development from the precontraction baseline was quantified. Figure 7A shows that the tension development in response to NO synthase (NOS) inhibition was greatest in Con (i.e.,  $\sim 55\%$  tension increase) vs. all other groups (i.e.,  $\sim 40\%$  tension increase). These findings indicate that NO bioavailability is blunted in all

Fig. 2. Representative tracing of intraluminal [NO] and vascular force from a coronary artery from a Con rat (A) and one with HHcy (B). BL, baseline.



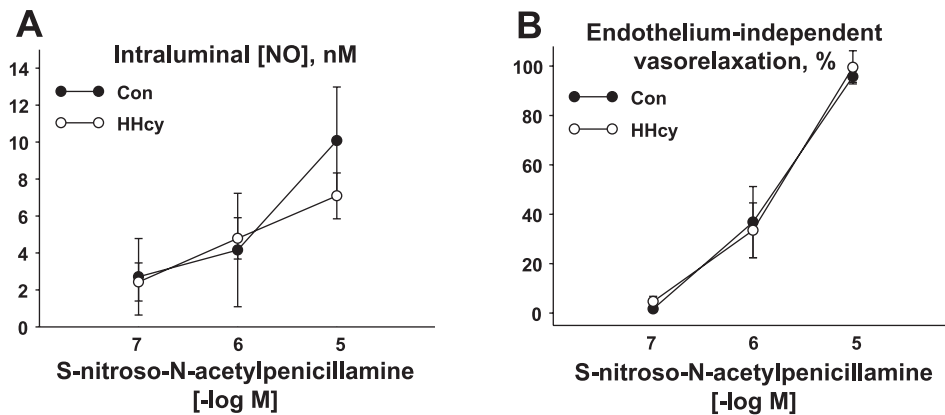


Fig. 3. Intraluminal [NO] (A) and vascular force (B) in coronary arteries from Con rats and those with HHcy in response to S-nitroso-N-acetylpenicillamine (SNAP). Values are means  $\pm$  SE.

groups vs. control animals. In the presence of L-NMMA, percent vasorelaxation in response to  $10^{-4}$  M ACh was inhibited to a similar degree among Con ( $9 \pm 9\%$ ), HHcy ( $1 \pm 2\%$ ), LF ( $1 \pm 1\%$ ), and HHcy + LF ( $4 \pm 3\%$ ) animals. These data verify the efficacy of NOS inhibition and demonstrate that ACh-evoked vasorelaxation is mediated primarily by NO in the rat aorta.

Differences among groups concerning endothelium-dependent vasorelaxation in aorta (i.e., Fig. 5A) were abolished after vessels were incubated for 30 min with the nonenzymatic intracellular  $O_2^-$  scavenger Tiron (Fig. 7B). In a subgroup of control rats, repeatability/time controls showed that maximal ACh-evoked vasorelaxation was  $92 \pm 15\%$  and  $90 \pm 2\%$  when concentration-relaxation response curves were separated by 30 min in the presence of the vehicle for Tiron.

Non-receptor-mediated (i.e., KCl) and receptor-mediated (i.e., NE) vasoconstrictive responses were similar among groups in aortic segments and mesenteric arteries (data not shown).

**Assessment of vascular  $O_2^-$ .** Vascular  $O_2^-$  was detected using DHE staining. DHE is a cell-permeant dye that emits blue fluorescence in the cytoplasm. In the presence of  $O_2^-$ , DHE is oxidized to ethidium, which emits red fluorescence. Ethidium is a DNA-binding fluorophore that is impermeable to intact cell membranes. The degree of red fluorescence is proportional to

the amount of intracellular  $O_2^-$  that is present. Relative fluorescence intensity (RFI) was 24–28% greater (see footnote 1 for explanation of range)<sup>1</sup> in tissue from HHcy + LF vs. all other groups (Fig. 7C; Fig. 8). Although not statistically

<sup>1</sup> Our results concerning DHE staining are expressed as a range of values because of a correction factor we employed that takes into consideration laser stability over time. In this regard, low and high standards were used to quantify stability of the laser and other acquisition/imaging parameters on each day of staining/imaging and over the time required to complete our study. The overall mean  $\pm$  SE for the 1) low standard was  $55.45 \pm 1.56$  RFI; 2) high standard was  $119.50 \pm 1.88$  RFI; and 3) sum of the low + high standards was  $174.95 \pm 1.72$  RFI. To calculate the normalization factor, the daily sum of the low + high standard RFI (e.g., 181.50) was divided by the overall sum of the low + high standard RFI (i.e., 174.95) to obtain a “normalization factor” ( $181.50/174.95 = 1.04$ ). Ideally, this normalization factor would be 1.0. Next, the “normalization factor” (i.e., 1.04) was multiplied by the RFI value obtained from tissue on that particular imaging day (e.g., 71.23) to provide a corrected value (e.g., 74.08). As mentioned earlier, the mean RFI was 24–28% greater in segments of thoracic aorta from HHcy + LF vs. Con animals. In fact, the mean “uncorrected” RFI of tissue from the HHcy + LF and Con groups was  $85.87 \pm 3.81$  and  $67.21 \pm 2.38$ , respectively, whereas the “normalized” RFI for the HHcy and Con groups was  $86.65 \pm 1.52$  and  $69.61 \pm 1.76$ , respectively. These values represent  $28 \pm 3\%$  (uncorrected) and  $24 \pm 2\%$  (corrected) increases in  $O_2^-$  production from thoracic aorta of HHcy + LF vs. Con animals. In both cases (e.g., corrected and uncorrected),  $O_2^-$  production was greater in tissue obtained from HHcy + LF vs. Con animals.

Table 1. Animal and vessel characteristics

	Con	HHcy	LF	HHcy + LF
<b>Animal characteristics</b>				
Age, days	98 $\pm$ 3	104 $\pm$ 4	98 $\pm$ 5	102 $\pm$ 4
No. Days on diet	68 $\pm$ 3	74 $\pm$ 4	68 $\pm$ 5	72 $\pm$ 4
Body weight, g	430 $\pm$ 13	379 $\pm$ 7	432 $\pm$ 7	388 $\pm$ 11
Heart rate, beats/min	385 $\pm$ 10	412 $\pm$ 18	415 $\pm$ 12	383 $\pm$ 10
Mean arterial pressure, mmHg	118 $\pm$ 3	119 $\pm$ 5	115 $\pm$ 4	116 $\pm$ 6
Glucose, mg/dl	113 $\pm$ 4	122 $\pm$ 3	120 $\pm$ 4	119 $\pm$ 4
Hematocrit, %	45 $\pm$ 1	45 $\pm$ 1	45 $\pm$ 1	47 $\pm$ 1
<b>Vessel characteristics</b>				
Aorta ID, $\mu$ m	1,702 $\pm$ 20	1,664 $\pm$ 19	1,642 $\pm$ 26	1,700 $\pm$ 89
Aortic medial thickness, $\mu$ m	86 $\pm$ 7	91 $\pm$ 10	90 $\pm$ 6	96 $\pm$ 10
Aorta length, $\mu$ m	3,459 $\pm$ 20	3,444 $\pm$ 61	3,348 $\pm$ 52	3,580 $\pm$ 129
Mesentery ID, $\mu$ m	191 $\pm$ 12	175 $\pm$ 9	172 $\pm$ 13	166 $\pm$ 8
Mesentery length, $\mu$ m	4,038 $\pm$ 116	4,122 $\pm$ 97	4,292 $\pm$ 103	4,233 $\pm$ 96
Coronary ID, $\mu$ m	150 $\pm$ 4	165 $\pm$ 9	160 $\pm$ 6	155 $\pm$ 4
Coronary length, $\mu$ m	2,250 $\pm$ 134	2,300 $\pm$ 128	2,075 $\pm$ 88	2,150 $\pm$ 128

Values are means  $\pm$  SE. Con, control; HHcy, hyperhomocysteinemia; LF, low folate. ID, internal diameter at 0-mg tension.

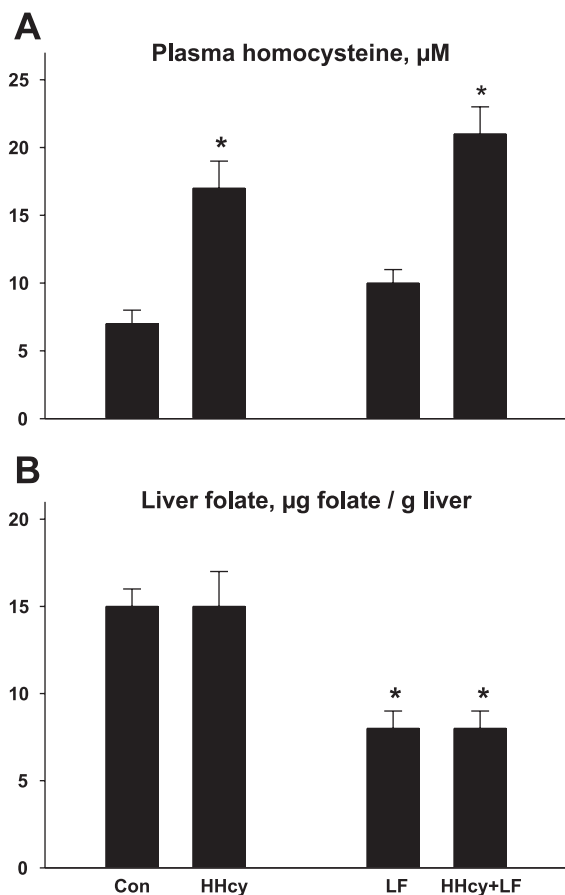


Fig. 4. Plasma homocysteine (A) was elevated similarly in HHcy and HHcy + low-folate (HHcy + LF) animals vs. both the Con and LF groups.  $*P < 0.05$ , HHcy vs. Con; HHcy + LF vs. LF. Liver folate (B) was reduced to the same extent in the LF and HHcy + LF groups compared with Con and HHcy animals.  $*P < 0.05$ , LF vs. Con; HHcy + LF vs. HHcy. Values are means  $\pm$  SE.

different, greater ( $P < 0.06$ ) RFI was observed in tissue from HHcy and LF vs. Con animals.

Seven control experiments for this analysis were performed. To verify that ethidium is a DNA-binding fluorophore, tissue sections were treated with DHE + 4,6-diamino-2 phenylindole dihydrochloride hydrate (DAPI; 20 nM in PBS). DAPI is a nuclear dye that fluoresces blue on binding to DNA. We observed 100% colocalization of DHE and DAPI, evidence that ethidium is localized to nuclei. Second, to discern whether DHE is selective for  $\text{O}_2^-$ , sections were pretreated with Tiron and then treated/incubated with DHE using procedures described earlier in METHODS. We observed an 85–100% attenuation of fluorescence in the presence vs. the absence of Tiron. Third, as a positive control, aortas and liver were obtained from a subgroup of rats 60 min after administration of the prooxidant agent diquat (dibromide monohydrate; 100  $\mu\text{mol/kg}$  ip) (1); compared with vehicle-treated rats, vascular staining for  $\text{O}_2^-$  and liver protein carbonyls and thiobarbituric acid reactive substances were elevated significantly. Fourth, to assess whether changes in optics/acquisition occurred over time, low and high standards (Molecular Probes, Eugene, OR) of known fluorescence were quantified before and after each staining/imaging session at the same settings that tissue sections were imaged. The minimal variation we observed (0.4%)

was accounted for and is detailed in footnote 1. Fifth, to determine consistency of the single observer we had for the present study, 320 nuclear areas from one animal per group were quantified on three separate days in a blinded manner. Less than 2% variability was observed among the 3 days for each group. Sixth, because cytochrome *c* released during apoptosis can convert DHE to ethidium (53) and because HHcy may initiate and/or contribute to apoptotic processes (22, 63), we compared caspase-3 activation (New England BioLabs, Beverly, MA) among groups. The percentage of vascular smooth muscle cells staining positive for caspase-3 vs. the total number of viable vascular smooth muscle cells was  $<2.5\%$  in the

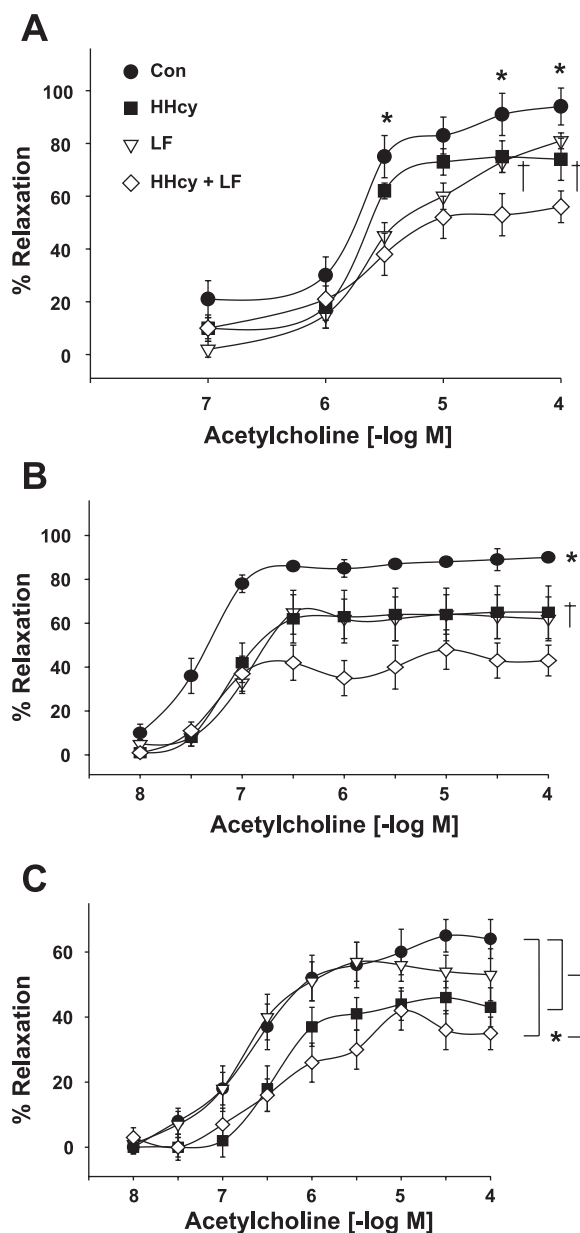


Fig. 5. ACh-evoked vasorelaxation was greatest in aortic segments (A) and mesenteric arteries (B) from Con animals vs. all groups. Vasorelaxation from the HHcy and LF groups was greater than observed in HHcy + LF animals.  $*P < 0.05$ , Con vs. all groups;  $\dagger P < 0.05$ , HHcy vs. HHcy + LF, LF vs. HHcy + LF. Coronary vasorelaxation in response to ACh (C) was greatest in vessels from Con vs. HHcy and HHcy + LF animals. Values are means  $\pm$  SE.

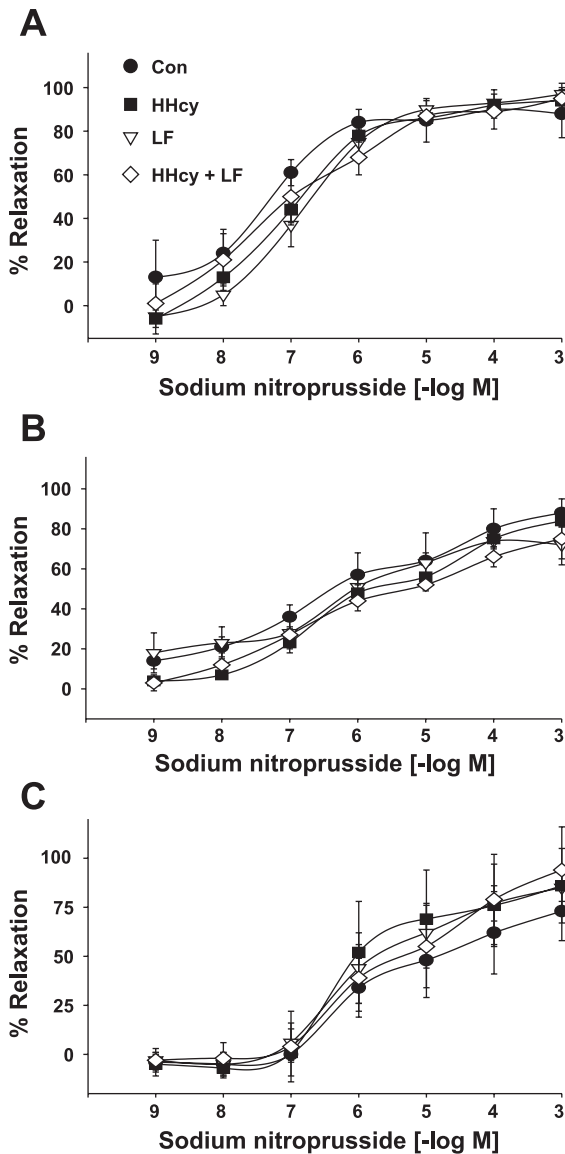


Fig. 6. Sodium nitroprusside-evoked vasorelaxation in aortas (A), mesenteric arteries (B), and coronary arteries (C) was similar among groups. Values are means  $\pm$  SE.

Con and HHcy + LF groups. Seventh, findings in the present study were confirmed using another quantification program (Volocity, Lexington, MA). Recent reports originating from the same laboratory subsequent to the collection of our data indicate that  $O_2^-$  generated in cultured endothelial cells in response to chemical and enzymatic stimuli reacts with DHE to form a fluorescent product that differs from ethidium, i.e., 2-hydroxyethidium (14, 65, 66). It remains to be elucidated whether this product is generated in vivo.

**Tissue indexes of oxidant load.** Protein carbonyls (nmol carbonyls/mg protein) were greater in liver from HHcy ( $1.77 \pm 0.08$ ), LF ( $1.74 \pm 0.05$ ), and HHcy + LF ( $1.70 \pm 0.10$ ) vs. Con animals ( $1.48 \pm 0.05$ ). Likewise, thiobarbituric acid reactive substances (nmol malondialdehyde/mg protein) were elevated in liver from HHcy ( $0.30 \pm 0.02$ ), LF ( $0.27 \pm 0.02$ ), and HHcy + LF ( $0.31 \pm 0.02$ ) vs. Con rats ( $0.23 \pm 0.02$ ). Cu-Zn + Mn SOD activity (U/mg protein) was greater in

aortic segments (combined from 3–4 animals from the same group) from HHcy ( $51.2 \pm 5.4$ ), LF ( $48.7 \pm 4.7$ ), and HHcy + LF ( $48.4 \pm 6.4$ ) vs. Con rats ( $36.5 \pm 5.2$ ).

## DISCUSSION

Findings from the present study indicate that HHcy and low folate independently impair arterial function and that HHcy-induced vascular dysfunction is most severe in the presence of low folate. Mechanisms responsible for these observations are that HHcy, LF, and their combination increase oxidant stress in general, and vascular  $O_2^-$  in particular, to an extent whereby

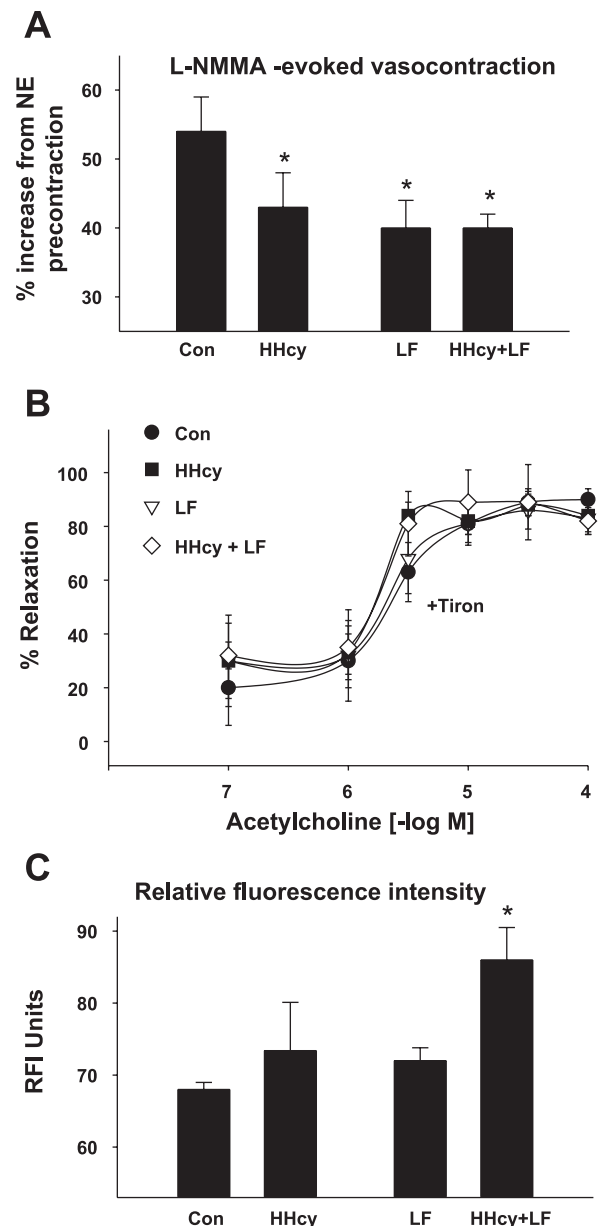


Fig. 7. A: vasocontraction in response to NO synthase inhibition using  $N^G$ -monomethyl-L-arginine (L-NMMA) was greatest in vessels from Con vs. all other groups. \* $P < 0.05$  vs. Con. NE, norepinephrine. B: ACh-evoked vasorelaxation was similar among groups after 30-min incubation with Tiron. C: vascular  $O_2^-$  production estimated by dihydroethidium (DHE) staining was greatest in aortas from HHcy + LF vs. all other groups. \* $P < 0.05$  vs. all groups. Values are means  $\pm$  SE. RFI, relative fluorescence intensity.

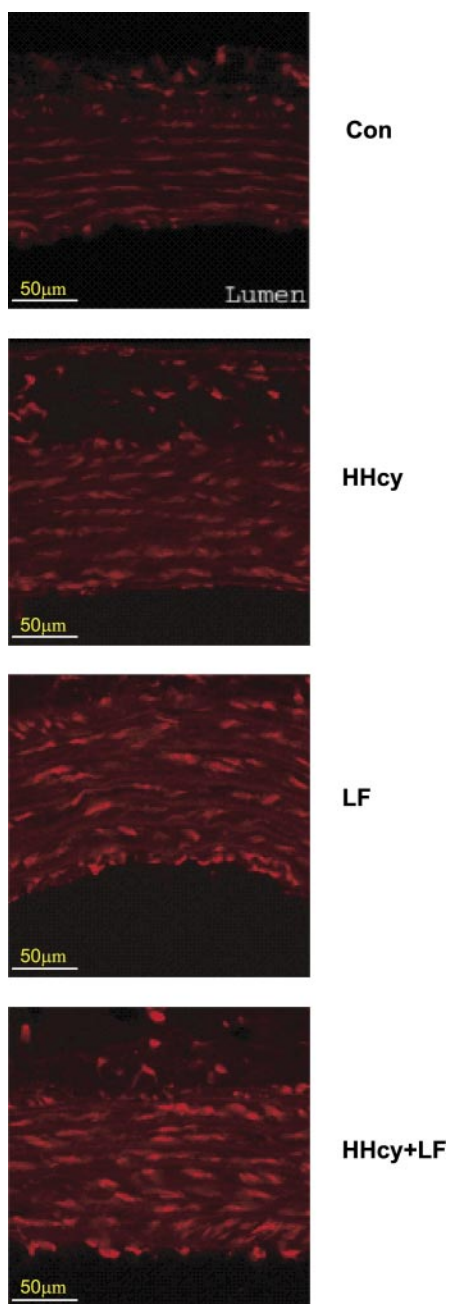


Fig. 8. Representative vessel from each group showing DHE staining for  $O_2^-$  production. Calibration bar, 50  $\mu\text{m}$ . Values are means  $\pm$  SE.

NO bioavailability is reduced. Furthermore, results obtained from aortic segments indicate that HHcy in combination with low folate evokes greater dysfunction than either HHcy or LF alone, likely by producing more  $O_2^-$  within the vasculature.

Plasma concentrations of homocysteine are controlled by two metabolic pathways: the remethylation and transsulfuration pathways. Remethylation of homocysteine requires the enzymes 5,10-methylenetetrahydrofolate reductase (5,10-MTHFR) and methionine synthase. Transsulfuration is dependent on cystathionine- $\beta$ -synthase (CBS) enzyme activity. Deficiencies in any of these enzymes and/or increased substrate for homocysteine metabolism elevates unmetabolized intracellular homocysteine, which is exported from the cell into the plasma. We used two

individual approaches, and their combination, to manipulate the remethylation pathway so that we could test our hypotheses. First, we produced pathophysiologically relevant HHcy by adding L-methionine to the drinking water. Second, we evoked  $\sim$ 50% reductions in liver folate by reducing dietary folate. Importantly, reductions in tissue folate were not sufficient to stimulate elevations of plasma tHcy. Third, L-methionine was added to the drinking water of rats that also consumed low-folate chow. In this group, an elevation of tHcy similar to HHcy animals was achieved in combination with a reduction in liver folate comparable to LF rats. Comparing results between the HHcy and HHcy + LF groups allowed us to test the hypothesis that HHcy-induced vascular dysfunction is most severe during low-folate conditions.

**HHcy and vascular function.** HHcy increases  $O_2^-$  and decreases bioavailable NO in a dose-dependent manner in endothelial cells (28, 56, 64). We confirmed and extended these findings by directly measuring intraluminal [NO] in coronary arteries (41, 43, 47). OxyHb-induced reductions in [NO] were less, and ACh-evoked increases in [NO] were blunted, in coronary vessels from HHcy vs. Con rats. Simultaneous measures of vascular force showed reduced OxyHb-induced tension development and blunted ACh-evoked vasorelaxation in coronary arteries from HHcy compared with Con animals. Taken together, these data indicate HHcy-induced alterations in intraluminal [NO] were functionally relevant.

Lower intraluminal [NO] in HHcy compared with Con vessels could result from reduced production/formation of NO and/or increased degradation of bioavailable NO. In the present study, we hypothesized the latter to be the most likely mechanism. This hypothesis was based partly on results from a previous study wherein HHcy-induced coronary dysfunction was not improved by excess extracellular L-arginine but was restored by Tiron or SOD + catalase (55). In the same report, increased protein 3-nitrotyrosine content was observed in coronary arteries from HHcy vs. control rats, indicating that dysfunction evoked by HHcy likely resulted from increased  $O_2^-$  formation and subsequent NO inactivation (55). We observed that HHcy caused dysfunction in the aortic, mesenteric, and coronary circulations. Dysfunction in the aorta was eliminated by Tiron, and vasoconstriction in response to NOS inhibition was  $\sim$ 40% less in aortas from HHcy than Con animals, suggesting  $O_2^-$  formation decreased NO bioavailability in aortas from HHcy rats. Moreover, aortic [i.e., Cu-Zn + Mn SOD activity and DHE fluorescence intensity ( $P < 0.06$ )] and overall (i.e., liver protein carbonyls and thiobarbituric acid reactive substances) oxidant stress were greater in HHcy vs. Con animals. Collectively, these findings provide further support for the hypothesis that HHcy evokes vascular dysfunction by increasing oxidant stress and decreasing NO bioavailability.

**Low folate and vascular function.** Most studies agree that exogenous folate improves vasorelaxation regardless of whether tHcy is lowered. Mechanisms proposed for improved endothelial function are that 5-MTHF has intrinsic antioxidant actions, improves NO production by endothelial NOS (eNOS), reduces  $O_2^-$  production by eNOS in the setting of compromised tetrahydrobiopterin ( $BH_4$ ) bioavailability, and reverses HHcy-evoked reductions in eNOS (38, 45, 57, 58, 64). Because of these findings concerning folate supplementation, it is not unreasonable to speculate that a critical decrease in folate would impair



NO bioavailability via decreased production and/or increased inactivation of this important vasoactive/antiatherosclerotic molecule. However, although reports indicate an association exists between low serum folate and impaired endothelial function in children (61), young men (21), older men (42), and patients with cardiovascular disease (3), the hypothesis that low folate impairs vascular function in an independent manner has never been tested. To answer this question, we restricted dietary folate to an extent that reduced liver folate concentrations by 50% but did not elevate tHcy. Liver folate was assessed because it represents long-term folate status, whereas serum/plasma and red blood cell measurements reflect short- and intermediate-term stores, respectively, and may be susceptible to fluctuations in metabolism (12, 15).

We observed that conductance (i.e., aorta) and resistance (i.e., mesentery) vessel function is compromised in rats with 50% reductions of liver folate, while coronary arteries are unaffected. Furthermore, because vasoconstriction in response to NOS inhibition was less and aortic and global indexes of oxidant load were greater in LF vs. Con rats, mechanisms responsible for LF-induced vascular dysfunction likely are similar to those described earlier for HHcy.

**Hyperhomocysteinemia + low folate and vascular function.** Results presented herein concerning the independent contributions from HHcy and LF provide proof of principle for our third hypothesis, i.e., that HHcy-induced vascular dysfunction is more severe in the presence of low folate. Further rationale is provided by two studies from the same laboratory. Dayal et al. (8) produced HHcy in CBS<sup>+/-</sup> mice by supplementing their drinking water with L-methionine for 7 (tHcy ~27  $\mu$ M) or 15 (tHcy ~24  $\mu$ M) wk [ $P < 0.05$  vs. 7 (tHcy ~6  $\mu$ M) or 15 (tHcy ~6  $\mu$ M) wk of standard water]. ACh-evoked vasorelaxation was depressed after 15 but not 7 wk. In an earlier investigation, Lentz et al. (30) produced a similar degree of HHcy (~25  $\mu$ M) in CBS<sup>+/-</sup> mice by feeding a folate-restricted diet. In that study, plasma folate was reduced by ~50% and aortic dysfunction was evident after 6 wk. Taking results from the two separate studies together, an identical degree of HHcy produced vascular dysfunction in the presence (30) but not the absence (8) of concomitant reductions in plasma folate. Although it is difficult to make conclusive statements by directly comparing results from two different investigations, these studies provide proof of principle for our hypothesis. We observed the greatest degree of vascular dysfunction in the combined presence of HHcy + LF. Specifically, maximal ACh-evoked vasorelaxation was ~24% (aortas), 34% (mesenteric arteries), and ~19% (coronary,  $P > 0.05$ ) less in vessels from HHcy + LF than HHcy rats. Greater resistance to the effects of HHcy + LF in the coronary circulation may be due to a number of factors, including differences concerning the local antioxidant environment and/or the ability of this important circulation to upregulate and/or compensate other endothelium-dependent vasodilatory mechanisms in response to reduced NO bioavailability.

HHcy-induced O<sub>2</sub><sup>-</sup> production was greater in the combined presence of low folate. One explanation for this is that eNOS can be converted from a NO-generating enzyme to a O<sub>2</sub><sup>-</sup>-producing enzyme in the presence of insufficient 5-MTHF. Specifically, BH<sub>4</sub> is required for converting L-arginine to L-citrulline + NO via eNOS (26, 27, 40). During this process, BH<sub>4</sub> is oxidized to quinoid dihydrobiopterin (qBH<sub>2</sub>) when it pro-

vides electrons for eNOS. Because 5-MTHF stimulates reduction of qBH<sub>2</sub> back to BH<sub>4</sub> (27), individuals lacking folate may have impaired resynthesis of BH<sub>4</sub>. Impaired synthesis of BH<sub>4</sub> results in eNOS binding to qBH<sub>2</sub> instead of BH<sub>4</sub>, which converts eNOS from a NO-generating enzyme to a O<sub>2</sub><sup>-</sup>-generating enzyme (24). This could explain why increased vascular O<sub>2</sub><sup>-</sup> production and greater dysfunction were observed in aortas from HHcy + LF vs. HHcy rats.

A more difficult finding to explain is that vasoconstriction after NOS inhibition was reduced similarly among treatment groups relative to Con animals. We expected L-NMMA-evoked vasoconstriction to be lower in aortas from HHcy + LF vs. HHcy or LF rats because O<sub>2</sub><sup>-</sup> production was greater. This was not observed. Instead, whereas stimulated endothelial release of NO (i.e., ACh-evoked vasorelaxation) was blunted to a greater degree in aortas from HHcy + LF vs. HHcy or LF animals, our estimate of basal NO release (i.e., tension development in response to NO synthase inhibition) was similar among treatment groups. It is unknown why the >24% increase in vascular O<sub>2</sub><sup>-</sup> production in aortas from HHcy + LF animals did not attenuate L-NMMA-evoked vasoconstriction to a greater degree vs. HHcy or LF rats.

We have shown that arterial vasorelaxation and NO bioavailability are compromised, and vascular O<sub>2</sub><sup>-</sup> production is exaggerated, in the setting of HHcy and low folate. NO bioavailability is important because in addition to preventing pathological vasoconstriction, endothelium-derived NO also inhibits platelet aggregation and leukocyte adhesion. Thus decreased bioavailability of NO is a plausible mechanism for increased risk of thrombosis and atherosclerosis in HHcy, and our data indicate this situation can be exacerbated in the presence of low folate. This scenario could have increased importance in elderly individuals. For example, tHcy increases with age (6), and low to low-normal concentrations or deficiencies of folate resulting from a number of factors (e.g., reduced intake, impaired absorption, interactions with medication) are not uncommon in the elderly (17, 34).

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