Vascular Signaling by Free Radicals
Oxidative stress-induced dysregulation of arteriolar wall shear stress and blood pressure in hyperhomocysteinemia is prevented by chronic vitamin C treatment

Zsolt Bagi,1,2 Csongor Cseko,2 Erika Tóth,2 and Akos Koller1,2
1Department of Physiology, New York Medical College, Valhalla, New York 10595; and 2Department of Pathophysiology, Semmelweis University, H-1445 Budapest, Hungary

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Bagi, Zsolt, Csongor Cseko, Erika Tóth, and Akos Koller. Oxidative stress-induced dysregulation of arteriolar wall shear stress and blood pressure in hyperhomocysteinemia is prevented by chronic vitamin C treatment. Am J Physiol Heart Circ Physiol 285: H2277–H2283, 2003.—We aimed to test the hypothesis that an enhanced level of reactive oxygen species (ROS) is primarily responsible for the impairment of nitric oxide (NO)-mediated regulation of arteriolar wall shear stress (WSS) in hyperhomocysteinemia (HHcy). Thus flow/WSS-induced dilations of pressurized gracilis muscle arterioles (basal diameter: ~170 μm) isolated from control (serum Hcy: 6 ± 1 μM), methionine diet-induced HHcy rats (4 wk, serum Hcy: 30 ± 6 μM), and HHcy rats treated with vitamin C, a known antioxidant (4 wk, 150 mg·kg⁻¹·day⁻¹; serum Hcy: 32 ± 10 μM), were investigated. In vessels of HHcy rats, increases in intraluminal flow/WSS-induced dilations were converted to contractions. Constrictions were unaffected by inhibition of NO synthesis by Nω-nitro-L-arginine methyl ester (L-NAME). Vitamin C treatment of HHcy rats reversed the WSS-induced arteriolar constrictions to L-NAME-sensitive dilations but did not affect control responses. Similar changes in responses were obtained for the calcium ionophore A-23187. In addition, diastolic and mean arterial blood pressure and serum 8-isoprostane levels (a marker of in vivo oxidative stress) were significantly elevated in rats with HHcy, changes that were normalized by vitamin C treatment. Taken together, our data show that in chronic HHcy long-term vitamin C treatment, by decreasing oxidative stress in vivo, enhanced NO bioavailability, restored the regulation of shear stress in arterioles, and normalized systemic blood pressure. Thus our data provide evidence that oxidative stress is an important in vivo mechanism that is primarily responsible for the development of endothelial dysregulation of WSS in HHcy.

SEVERAL EPIDEMIOLOGICAL STUDIES have shown that hyperhomocysteinemia (HHcy) increases the risk for cardiovascular diseases, such as ischemic heart diseases; cerebrovascular, peripheral vascular diseases; and hypertension (1, 6, 7, 9, 29, 32). Homocysteine is formed during the metabolism of the essential amino acid methionine, and its normal plasma concentration is between 5 and 15 μM, but it can be increased due to genetic (e.g., cystathione-β-synthase and methylenetetrahydrofolate reductase) and nutritional alterations (deficiency of vitamins, e.g., folic acid, vitamin B6, and B12), factors that participate in the metabolism of homocysteine and methionine (8).

Although the underlying mechanisms responsible for the elevated risks have not yet been fully elucidated, there is increasing evidence to suggest that endothelial dysfunction of vessels contributes to the development of vascular diseases observed in both humans and animals with HHcy (19, 34). Several studies have documented that even moderate HHcy (15–30 μM) is associated with a significant impairment of endothelium-dependent relaxation of large vessels (20, 28) and dilation of arterioles (3, 31), primarily due to the reduced mediation of responses by nitric oxide (NO). The nature of mechanisms by which elevated levels of plasma homocysteine elicits reduction in NO-mediation of vascular responses, however, is still not completely understood.

In an isolated aortic ring preparation of HHcy rabbits impaired endothelium-dependent relaxations could be restored by acute administration of vitamin C to the vessel chamber (18). Similarly, acute in vitro administration of superoxide dismutase (a scavenger of superoxide) or inhibition of reactive oxygen species (ROS)-producing enzymes restored flow-induced, NO-mediated dilations of coronary or skeletal muscle arterioles of HHcy rats (2, 30). From these and other studies, it has been hypothesized that ROS play an important role in the development of vascular dysfunction in HHcy.

Although previous studies suggest an important role for ROS in the development of HHcy-induced alterations in endothelial function, less evidence is provided whether reduction of oxidative stress in vivo can pre-
vent and/or reduce the development of HHcy-induced endothelial dysfunctions (13, 18, 21, 35). Recent human studies have shown 1) that oral methionine load-induced, transient HHcy elicited impairment of brachial artery dilation in response to forearm occlusion (4, 5, 14) and dilation of resistance vessels to ACh (25) and 2) that these impairments could be prevented by prior oral administration of vitamin C or vitamin E. Also, in severe HHcy patients with homocystinuria, vitamin C treatment improved similar responses (24), although the mechanisms of action of vitamin C remain obscure in these studies. Interestingly, in contrast to these findings, Hanratty et al. (10) have found that prior administration of vitamin C did not prevent the impairment of endothelium-dependent dilation to ACh of forearm vessels after acute administration of methionine. Moreover, others (17, 22) did not even find endothelial dysfunction in conduit and resistance vessels after acute oral methionine load. These discrepant findings do not allow one to draw conclusions regarding the causative role of oxidative stress in the development of vascular dysfunction in HHcy. In addition, there are studies that link HHcy to hypertension (26, 27), but the findings are also contradictory (32). Also, the potential role of dysfunction of wall shear stress (WSS) in the possibly enhanced vascular resistance has not yet been clarified.

Thus it seems to be important to test the hypothesis that a pathophysiological relevant level of oxidative stress is indeed present in HHcy in an otherwise healthy vascular system, and its reduction by chronic use of antioxidant prevents the development of endothelial dysregulation of arteriolar WSS, a local mechanism known to be importantly involved in the regulation of peripheral vascular resistance, and hence blood pressure.

**MATERIALS AND METHODS**

*Experimental design.* Male Wistar rats (n = 60) weighing 150 g and purchased from Charles River Laboratories (Wilmington, MA) were housed separately, had free access to water, and fed standard rat chow. All protocols were approved by the Institutional Animal Care and Use Committees at New York Medical College and Semmelweis University. At the age of 6 wk, animals were randomly separated into four groups. In two groups of animals (n = 15/15), moderate HHcy was induced by daily administration of L-methionine (1 g·kg body wt⁻¹·day⁻¹) in the drinking water for a period of 4 wk (17, 22). Another two groups of rats received normal drinking water, and they served as controls (n = 15/15). One of the control (n = 15) and HHcy (n = 15) groups were treated with vitamin C (150 mg·kg body wt⁻¹·day⁻¹) by daily oral gavage.

*Measurement of arterial blood pressure and determination of serum total homocysteine and 8-isoprostane levels.* After overnight fasting, rats were anesthetized with an intraperitoneal injection of ketamine-xylazine (80:12 mg/kg). A MicroTip pressure catheter transducer (model SPR-524 connected to model TCB-600 pressure control unit; Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the aorta to measure systolic and diastolic arterial blood pressure. Blood was then collected from the carotid artery, immediately cooled on ice, and centrifuged at 3,000 g for 10 min at 4°C. Until chemical assays, serum was stored at −80°C. Total serum homocysteine concentrations were measured by a commercial homocysteine enzyme immunoassay kit (Abbott Laboratories; N. Chicago, IL). Serum levels of free 8-isoprostane were measured by a commercial enzyme immunoassay kit (Cayman Chemical; Ann Arbor, MI).

**Isolation of arterioles.** Experiments were conducted on isolated arterioles (inside diameter: ~170 μm) of rat gracilis muscle as described previously (16). Briefly, during anesthesia and with the use of microsurgery instruments and an operating microscope, the gracilis muscle was exposed and isolated from surrounding tissues. A segment, ~1.5 mm in length of an arteriole running intramuscularly, was isolated and transferred into an organ chamber containing two glass micropipettes filled with physiological salt solution (PSS). Arterioles were then cannulated on both sides in an organ chamber and were continuously superfused with PSS (in mM: 110 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 1.0 K₂HPO₄, 5.0 glucose; and 24.0 NaHCO₃ equilibrated with 10% O₂:5% CO₂:85% N₂ at pH 7.4). Inflow and outflow pressures were controlled by a pressure servocontrol system (Living System Instrumentation; Burlington, VT). The temperature was set at 37°C by a temperature controller, and the vessel was allowed to develop spontaneous tone in response to 80-mmHg intraluminal pressure under no flow conditions (equilibration period: 1 h). The inside diameter of arterioles was measured by videomicroscopy (16), recorded with a Biopac MP100 system connected to a computer, and analyzed with AcqKnowledge data-acquisition software (Biopac Systems; Goleta, CA).

*Arterial responses to ACh, A-23187, and sodium nitroprusside.* After the equilibration period, peak arterial responses were obtained to cumulative doses of ACh (10⁻³–10⁻⁶ M), calcium ionophore A-23187 (3 × 10⁻⁹–3 × 10⁻⁸ M), and sodium nitroprusside (SNP; 10⁻⁹–10⁻⁶ M). Vessels were then incubated with the NO synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 10⁻⁴ M, for 30 min). In separate experiments, thromboxane A₂ receptor antagonist SQ-29548 (10⁻⁶ M, for 20 min) was used. After inhibitors, agonist-induced arterial responses were again obtained.

*Flow/WSS-induced responses.* In separate experiments, changes in diameter of arterioles were measured in response to step increases in intraluminal flow (0–30 μl/min), eliciting increases in WSS. Intraluminal flow was established at a constant intravascular pressure (80 mmHg) by changing the inflow and outflow pressure to an equal degree but in opposite directions to keep midpoint luminal pressure constant. Flow was measured with a ball flowmeter (Omega Engineering; Stamford, CT). Each flow rate was maintained for 5 min to allow the vessel to reach a steady-state diameter, and at this time, WSS was calculated. The vessels were then incubated with L-NAME (10⁻⁴ M), and flow/WSS-induced arterial responses were obtained again.

*Materials.* All drugs were added to the vessel chamber and final concentrations are reported. All salts and chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise mentioned, and solutions were prepared on the day of the experiment.

*Data analysis.* Basal tone of arterioles was calculated from the active (AD; in Ca²⁺-containing PSS) and passive diameters (PD; in Ca²⁺-free PSS containing 10⁻³ M EGTA and 10⁻⁴ M SNP) at 80-mmHg pressure as follows: ([PD − AD/PD] × 100) expressed as a percent. Agonist-induced dilations
were expressed as a percentage of the passive diameter. Constrictions were expressed as a percentage of baseline diameters. WSS values were calculated according to the formula: WSS = 4ηQ/r², where η is the viscosity of the perfusate (0.007 poised at 37°C), Q is the perfusate flow, and r is the vessel radius (15). Statistical analyses were performed by two-way, repeated-measures ANOVA followed by Tukey’s post hoc test or Student’s t-test, as appropriate. P < 0.05 was considered statistically significant. Data are expressed as means ± SE.

RESULTS

There was no significant difference between the body weight of control and HHcy rats, and it was also unaffected by vitamin C treatment (Table 1). A methionine-rich diet induced a significant increase in serum total homocysteine concentration. Vitamin C treatment had no effect on serum homocysteine concentrations of rats either in HHcy or control groups (Table 1). Compared with controls, HHcy rats were characterized by a significant increase in diastolic and mean blood pressure, without significant change in systolic pressure. Vitamin C treatment significantly decreased diastolic and mean blood pressures of HHcy rats but did not affect those of control rats (Table 1).

Serum level of 8-isoprostane. Compared with controls, serum levels of 8-isoprostane (a marker of oxidative stress) (23) were significantly higher in HHcy rats and were reduced by vitamin C treatment (Fig. 1A). Vitamin C treatment did not significantly change the serum levels of 8-isoprostane in control animals (Fig. 1A).

Arterial responses to agonists. Arterioles isolated from gracilis muscle of control and HHcy rats developed active tone in response to an intraluminal pressure of 80 mmHg without the use of any vasoactive agent. There was no significant difference between passive diameters (in the absence of extracellular Ca²⁺) and active diameters of arterioles of various groups and the calculated basal tone in vessels of various groups of animals (Table 1).

To document the HHcy-induced endothelial dysfunction, arteriolar responses were obtained by using vasoactive agents with a known mechanism of action. In a dose-dependent manner, the NO donor SNP elicited dilations of arterioles, which were not significantly different in control, HHcy-, and vitamin C-treated groups and the calculated basal tone in various groups of animals (Table 1).
groups of animals (Fig. 1B). In contrast, ACh elicited significantly reduced peak dilations of HHcy arterioles compared with those of control arterioles (Fig. 2A). In arterioles of vitamin C-treated HHcy animals, the reduced ACh-induced dilations were significantly enhanced, whereas vitamin C treatment had no effect on responses of control arterioles (Fig. 2A). Inhibition of endothelial NO synthase (eNOS) by L-NAME significantly reduced ACh-induced dilations in control, vitamin C-treated control, and vitamin C-treated HHcy arterioles, but it did not affect ACh-induced constrictions of HHcy vessels (Fig. 2D).

Flow/WSS-induced arteriolar responses. Stepwise increases in WSS elicited by increases in intraluminal flow (0–30 μl/min) resulted in substantial dilations of arterioles isolated from control rats but constricted arterioles isolated from HHcy animals (Fig. 3A). Inhibition of eNOS by L-NAME significantly reduced (≈50%) shear stress-induced dilations in control arterioles, but it had no effect on shear stress-induced constriction of HHcy arterioles (Fig. 3A). Vitamin C treatment did not affect responses of control arterioles, whereas it restored shear stress-induced dilations of arterioles isolated from vitamin C-treated HHcy rats (Fig. 3B). Inhibition of eNOS by L-NAME significantly reduced shear stress-induced dilations of arterioles of vitamin C-treated control and HHcy rats (Fig. 3B).

Figure 3C shows that at the maximum flow rate (30 μl/min) in control arterioles, the peak values of WSS were ~4 dyn/cm², whereas they were ~10 dyn/cm² in
HHcy arterioles. In HHcy vessels, vitamin C treatment significantly decreased the peak WSS values to near the control level (Fig. 3).

**DISCUSSION**

The new findings of this study are that in the 4-wk model of rat methionine diet-induced HHcy, vitamin C treatment 1) decreased 8-isoprostane (a marker of in vivo oxidative stress) levels without affecting the elevated concentration of plasma homocysteine, 2) restored NO-mediation of WSS-induced dilations of skeletal muscle arterioles, and 3) normalized diastolic and mean arterial blood pressures. These findings provide in vivo evidence that in chronic HHcy, oxidative stress is primarily responsible for the development of arteriolar dysregulation of WSS and elevation of blood pressure.

**Altered regulation of arteriolar WSS and its systemic consequences.** One of the primary targets of HHcy by which it may increase the risk of cardiovascular diseases (1, 6, 9, 29) is altering the endothelial function of vessels, because a positive correlation was found between endothelial dysfunction and elevated cardiovascular risk in HHcy (19, 34). Earlier studies have demonstrated that even moderate HHcy is associated with significant impairment of endothelium-dependent dilations of large vessels (20, 28) and arterioles (3, 31). Endothelial dysfunction is often characterized by decreased vasodilations induced by pharmacological agonists and physiological stimuli (e.g., increases in flow/shear stress), and in many of studies in HHcy, a decreased NO-mediation of dilator responses was demonstrated.

In the present study, we first confirmed our previous finding (3) that HHcy impairs NO-mediation of skeletal muscle arteriolar dilations. In addition, we have found that the calcium ionophore A-23187 (12), which induced significant L-NAME-sensitive dilations in control arterioles, in HHcy arterioles elicited constrictions due to the simultaneous lack of NO mediation and enhanced thromboxane A2 production as we have found similarly with increases in flow (3). These findings suggest that NO and prostaglandin mediations of responses are affected in HHcy regardless of whether the stimulus is receptor dependent or independent, adding further support to our previous findings (3).

In the present study, we further characterized the effect of increases in intraluminal flow on arteriolar diameter in HHcy by calculating WSS. These data show that increases in WSS, instead of dilation, elicited constrictions of skeletal muscle arterioles of HHcy rats (Fig. 3A). These constrictions resulted in an ∼2.5-fold increase in maximum WSS in gracilis skeletal muscle arterioles of rats with HHcy (Fig. 3C). Dysregulation of WSS (higher WSS values for the same flow rate, as shown in Fig. 3C) in resistance arterioles could lead to increased power dissipation in the circulation with the consequent adaptive elevation of blood pressure (to maintain adequate cardiac output) unless other mechanisms compensate. Thus dysregulation of WSS could contribute to the significant elevation in diastolic and mean arterial blood pressures in 4-wk HHcy (Table 1). Previous population-based clinical studies have shown a positive correlation between fasting plasma homocysteine level and blood pressure without revealing the underlying mechanisms (27). The present findings suggest a pathological mechanism that provides a possible link between HHcy and development of hypertension. Thus we propose that in HHcy impairment of endothelium-mediated regulation of arteriolar WSS due to an increased circulatory power dissipation is likely to be responsible, at least in

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Fig. 3. Changes in diameter of gracilis skeletal muscle arterioles isolated from normal (control) and HHcy rats (n = 9 rats/group; A) and Control + Vit C, and HHcy + Vit C rats (n = 7 rats/group; B) before and after incubation of L-NAME as a function of increases in wall shear stress (WSS). C: changes in WSS of arterioles isolated from control, HHcy, Control + Vit C, and HHcy + Vit C rats as a function of increases in intraluminal flow. Data are means ± SE. *Significant differences at P < 0.05.
part, for the observed increase in blood pressure, although cardiac alterations or other mechanisms may also contribute (26, 32).

Role of in vivo oxidative stress in HHcy-induced arteriolar impairment. On the basis of in vitro studies, it has been assumed that oxidative stress plays an important role in the development of HHcy-induced vascular dysfunction (13, 21, 35). Indeed, it seemed to be an attractive hypothesis that increased in vivo production and/or level of ROS, primarily superoxide anion, interacting with vascular signaling mechanisms (such as NO mediation) may contribute to the development of vascular dysfunction observed in HHcy. However, previous studies (2, 18) suggesting the role of oxidative stress in chronic HHcy-induced vascular dysfunctions were mostly based on experiments using acute administration of antioxidants in vitro. For example, we have found that in isolated skeletal muscle arterioles of HHcy rats, reducing arteriolar superoxide level by acute in vitro administration of superoxide dismutase or pharmacological inhibition of superoxide-producing vascular enzyme xanthine oxidase restored NO and prostaglandin mediation of arteriolar dilations (2). As we have shown previously, the enhanced level of superoxide by acute administration of superoxide dismutase or pharmacological inhibition of superoxide-producing vascular enzyme xanthine oxidase restored NO and prostaglandin mediation of arteriolar dilations (2). As we have shown previously, the enhanced level of superoxide by acute administration of superoxide dismutase or pharmacological inhibition of superoxide-producing vascular enzyme xanthine oxidase restored NO and prostaglandin mediation of arteriolar dilations (2).

Only a few reports have provided data for the possible in vivo role of oxidative stress in the development of HHcy-induced vascular dysfunction (10, 24), yet these studies are conflict (30) regarding the nature of vascular alterations and effectiveness of antioxidant treatments. These conflicting results, at least in part, may be due to the fact that human studies have a number of limitations, such as other unrevealed pathological conditions, ongoing treatments, and methodological limitations. Thus it was necessary to test the hypothesis that long-term administration of vitamin C via reduction of in vivo oxidative stress prevents the development of arteriolar endothelial dysfunction in HHcy. These studies are important to provide evidence for the in vivo role of oxidative stress in HHcy-induced microvascular dysfunction, especially to regulation of WSS.

In the present study, 4 wk of methionine-rich diet (2) elicited a moderate increase in serum levels of total homocysteine (Table 1), comparable with the human form of this disease (8). Vitamin C treatment did not affect the serum concentration of total homocysteine either in control or in HHcy rats (Table 1) but significantly reduced the elevated serum levels of 8-isoprostane in HHcy animals (Fig. 1A). It is known that 8-isoprostanes are a family of eicosanoids of nonenzymatic origin produced in vivo by the random oxidation of tissue phospholipids by oxygen free radicals providing a reliable marker of in vivo oxidative stress (23). Importantly, a positive correlation was found between plasma homocysteine and F2-isoprostane concentrations in humans (33). Lowering serum 8-isoprostane levels by long-term vitamin C treatment in HHcy rats found in this study provides evidence for the idea that vitamin C in vivo effectively scavenges oxygen free radicals and thus decreases oxidative stress in HHcy.

We found that vitamin C had no affect on NO mediation of agonist- and flow/shear stress-induced dilations in arterioles of control rats (Figs. 2 and 3). However, despite the presence of elevated serum concentration of homocysteine, vitamin C treatment enhanced NO mediation of ACh- and A-23187-induced arteriolar dilations in HHcy, restoring them close to the magnitude of control responses (Fig. 2). More importantly, vitamin C treatment converted WSS-induced constrictions to dilations in HHcy arterioles. Because the restored dilations could be significantly reduced by L-NAME (Fig. 3), we concluded that vitamin C prevented the impairment of NO-mediated regulation of WSS in HHcy. Furthermore, the restored regulation of arteriolar WSS was accompanied by normalization of diastolic and mean arterial blood pressures in HHcy animals. Collectively, these findings suggest a causative role for in vivo enhanced level of ROS in the development of NO- and prostaglandin-mediated endothelial dysfunction and consequent dysregulation of arteriolar WSS in HHcy. Furthermore, our results also suggest a possible underlying mechanism for the blood pressure lowering effect of the antioxidant vitamin C in HHcy similarly to other vascular disorders associated with oxidative stress (11). In addition, these results demonstrate the beneficial vascular effects of vitamin C treatment in HHcy and provide rational for its clinical use as efficient antioxidant with cardiovascular protection.

In conclusion, we have demonstrated that long-term treatment of HHcy rats with the antioxidant vitamin C, by reducing in vivo oxidative stress, restored NO- and prostaglandin-mediated regulation of shear stress in resistance arterioles (an important mechanism aiming to reduce circulatory power dissipation) and normalized systemic blood pressure. Thus our study provides important evidence for the causative role of oxidative stress in the development of endothelial dysregulation of shear stress in HHcy and a rational for therapeutic use of vitamin C, especially in diseases resistant to interventions aiming to reduce elevated plasma homocysteine level.

DISCLOSURES
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