Xanthine Oxidase–Derived Reactive Oxygen Species Convert Flow-Induced Arteriolar Dilation to Constriction in Hyperhomocysteinemia

Possible Role of Peroxynitrite

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Abstract—We hypothesized that in hyperhomocysteinemia (HHcy), flow-induced arteriolar constriction is due to an enhanced generation of reactive oxygen and/or nitrogen species, causing an impairment of nitric oxide (NO) and prostaglandin mediation of the response. Changes in diameter of isolated, pressurized (at 80 mm Hg) gracilis muscle arterioles (diameter = 170 μm) from control and methionine diet–induced HHcy rats were measured by videomicroscopy. Increases in intraluminal flow (from 0 to 25 μL/min) resulted in significant NO- and prostaglandin-mediated dilations of control arterioles (maximum, control, 30±4 μm) but elicited significant constrictions of HHcy arterioles (maximum, HHcy, −32±3 μm), which were abolished by the thromboxane A2 receptor blocker SQ 29,548. Intraluminal administration of superoxide dismutase plus catalase did not affect flow-mediated dilations of control arterioles, but in HHcy arterioles, it reversed the flow-induced constrictions to dilations (maximum 18±4 μm), which were abolished by an NO synthase inhibitor. Flow-induced constrictions of HHcy arterioles were prevented by the presence of the xanthine oxidase inhibitor oxyxpurinol [but not by the NAD(P)H-oxidase inhibitor diphenyleneiodonium] and by urate, a known peroxynitrite scavenger. Also, authentic peroxynitrite elicited arteriolar constrictions (−31±8 μm) that were eliminated by urate and SQ 29,548. Thus, we suggest that in HHcy, xanthine oxidase–derived superoxide scavenges NO released to flow, forming peroxynitrite, which promotes release of thromboxane A2, resulting in arteriolar constriction.


Key Words: homocysteine • arterioles • flow-induced constriction • thromboxane A2 • reactive oxygen species • xanthine oxidase

Increased plasma concentration of the methionine metabolite homocysteine1 is an independent risk factor for cardiovascular diseases, such as stroke, myocardial infarction, peripheral vascular disease, and atherosclerosis.1,2 One of the mechanisms by which hyperhomocysteinemia (HHcy) elicits its adverse vascular effects is by altering endothelial function. Recent studies by other laboratories and ours showed that even mild HHcy (15 to 30 μmol/L) is associated with significant impairment of endothelium-dependent acetylcholine-, histamine-, or bradykinin-induced vasoactive responses in large arteries and aorta of monkeys3,4 and mice,5–7 as well as in rat skeletal muscle arterioles.8,9 Furthermore, we demonstrated that increases in intraluminal flow–related wall shear stress, the primary stimulus for the endothelial synthesis/release of nitric oxide (NO) and prostaglandins (PGs) in vivo, which maintain a dilated state of arterioles, elicit constrictions in arterioles of HHcy rats because of the simultaneous lack of NO mediation and enhanced thromboxane A2 (TXA2) release.10 These findings have important clinical applications, because in humans, a similar degree of mild HHcy, either basal or transient after a methionine load, is associated with an impaired dilation or even constriction of arteries in response to a release of forearm occlusion.11–16 The underlying mechanisms responsible for flow-induced endothelium-dependent constriction in HHcy, however, remained unknown.

Flow-dependent skeletal muscle, arteriolar dilation is mediated by NO and dilator PGs, the synthesis/release of which can be substantially affected by reactive oxygen species (ROS).17 Studies on endothelial cells in culture suggest that high levels of homocysteine may promote the generation of ROS.18–22 Previous studies demonstrated that ROS [in particular, superoxide produced by endothelial NAD(P)H oxidase and/or xanthine oxidase] may impair arteriolar functions in pathophysiological conditions, such as hypertension,23 hypercholesterolemia,24 and diabetes.25 A role for oxidative stress in HHcy-induced endothelial dysfunction in humans is also supported by recent studies showing that oral administration of antioxidants (eg, vitamin C, vitamin E) prevented oral methionine load–induced impairment of dilation of conduit arteries to acetylcholine26 and after release of forearm occlusion.27,28
On the basis of the aforementioned studies, we hypothesized that in HHcy, an enhanced generation of ROS alters NO and PG mediation of flow-induced arteriolar dilations. Thus, to test this hypothesis, flow-induced responses in isolated skeletal muscle arterioles of HHcy rats were investigated in the presence and absence of pharmacological probes of known action on synthesis of endothelial factors and metabolism of free radicals.

**Methods**

Male Wistar rats (n=60, 300 to 330 g, purchased from Charles River Co, Budapest, Hungary) were housed separately and had free access to water and standard rat chow. In 1 group (n=30), moderate HHcy was induced by administration of L-methionine (1 g · kg body wt·d⁻¹) in the drinking water for a period of 4 weeks. The doses of L-methionine administered were based on average daily fluid intake. Previous studies and our present findings show that this methionine-rich diet increases plasma homocysteine concentration 3-fold in rats. 9,29,30

**Isolation of Arterioles**

Experiments were conducted on isolated arterioles (inside diameter 170 μm) of rat gracilis muscle as described previously. 9,9 Arterioles were cannulated on both sides in an organ chamber and were continuously superfused with physiological salt solution (in mmol/L: NaCl 110, KCl 2.5, MgSO₄ 1.0, KH₂PO₄ 1.0, glucose 10.0, and NaHCO₃ 24.0; equilibrated with 10% O₂, 5% CO₂, 85% N₂, at pH 7.4). Inflow and outflow pressures were measured by an electromanometer. The temperature was set at 37°C by a temperature controller (Grant Instruments), and the vessel was allowed to develop spontaneous tone in response to intraluminal pressure under no-flow conditions (equilibration period 1 hour). The inner arteriolar diameter was measured by videomicroscopy. 3–10 Intraluminal flow was established at a constant intravascular pressure (80 mm Hg) by changing the inflow and outflow pressure to an equal degree but in opposite directions to keep midpoint luminal pressure constant and was measured with a ball flowmeter (Omega Engineering Inc).

**Experimental Protocols**

After the equilibration period, changes in diameter of arterioles were assessed in response to step increases in intraluminal flow (from 0 to 25 μL/min). Then arterioles were exposed in various protocols to incubation with the TXA₂ receptor antagonist SQ 29,548 (10⁻⁸ mol/L for 20 minutes), or the free radical scavenger superoxide dismutase (SOD, 120 U/mL intraluminally for 30 minutes), 17 or catalase (CAT, 80 U/mL intraluminally for 30 minutes), or SQ 29,548 (plus SOD/CAT), or the cyclooxygenase inhibitor indomethacin (10⁻⁵ mol/L, plus SOD/CAT), or the NOS inhibitor L-NAME, methyl ester (L-NAME, 10⁻⁴ mol/L, plus SOD/CAT), or the NO synthase (NOS) substrate L-arginine (10⁻⁴ mol/L), or the peroxynitrite (ONOO⁻) scavenger urate (10⁻⁴ mol/L, for 30 minutes), or urate plus SOD, or the NAD(P)H-oxidase inhibitor diphenyleneiodonium (DPI, 3×10⁻⁴ mol/L), 17 or the xanthine oxidase inhibitor oxypurinol (10⁻⁴ mol/L), 17 and flow-induced responses were obtained again. In separate experiments, constant flow (20 μL/min) was established in HHcy arterioles, and responses to arachidonic acid (10⁻⁵ mol/L) were tested, followed by administration of SQ 29,548. Responses of control arterioles to increasing concentrations of authentic ONOO⁻ were also obtained in the absence and presence of SQ 29,548 or urate. At the conclusion of each experiment, the maximal passive dilation was obtained after incubation of arterioles in a Ca²⁺-free solution that contained EGTA.

**Materials**

Authentic ONOO⁻ was obtained from Calbiochem as an aqueous stock solution in 4.7% NaOH (nominal concentration 200 mmol/L) and was stored in aliquots at −80°C under inert gas, protected from light and air. Immediately before each experiment, an aliquot of the stock solution was diluted into an ice-cold alkaline solution, 32 from which an appropriate volume was administered immediately in the organ bath. SQ 29,548 (Cayman Chemicals) was dissolved in ethanol; urate (Calbiochem) was dissolved in alkaline buffer. The vehicle did not have a vasoactive effect. All other salts and chemicals were obtained from Sigma-Aldrich Co, and solutions were prepared on the day of the experiment.

**Data Analysis**

Changes in arteriolar diameter are expressed as absolute values. Statistical analyses were performed by 2-way ANOVA for repeated measures followed by the Tukey post hoc test or Student’s t test, as appropriate. A value of P<0.05 was considered statistically significant. Data are expressed as mean±SEM.

**Results**

There was no significant difference between body weight (control 325±15 g and HHcy 314±21 g) and daily water intake of control and methionine-fed rats. Arterioles isolated from gracilis muscle of control and HHcy rats developed active tone in response to intraluminal pressure of 80 mm Hg without the use of any vasoactive agent (control 179±7 μm and HHcy 174±15 μm, P=NS). The passive diameter of control and HHcy arterioles in the absence of extracellular Ca²⁺ were 247±17 and 237±15 μm (P=NS), respectively (at 80 mm Hg). There was no significant difference in basal arteriolar diameter either between the control and HHcy groups or after administration of various enzyme inhibitors and receptor antagonists in zero-flow conditions.

**Role of TXA₂ in Flow-Induced Constriction**

Stepwise increases in intraluminal flow elicited significant dilations in control arterioles but constricted HHcy arterioles. In HHcy arterioles, the flow-induced constrictions were completely abolished in the presence of the PGH₂/TXA₂ receptor antagonist SQ 29,548, which did not affect dilations of control arterioles (Figure 1A). Figure 1B shows a bioassay experiment on an HHcy arteriole. In the presence of intraluminal flow, administration of arachidonic acid elicited substantial constriction (32±6 μm, n=4). Subsequent administration of SQ 29,548 completely reversed responses to arachidonic acid and normalized arteriolar diameter to baseline (preflow) levels.

**Effects of Free Radical Scavengers**

To provide evidence for the role of endothelium-derived ROS in flow-induced constrictions, SOD and CAT were administered intraluminally, a method that was shown to effectively scavenge superoxide generated by high intraluminal pressure in skeletal muscle arterioles. 17 The presence of SOD plus CAT or SOD alone converted flow-induced constrictions of HHcy arterioles to dilations (maximum 18±4 and 15±3 μm, respectively) but did not affect flow-induced dilations of control arterioles (Figure 1C). SOD plus CAT—converted flow-induced dilations of HHcy arterioles were not affected by indomethacin or SQ 29,548 (Figure 2A) but were abolished by the NOS inhibitor L-NAME (Figure 2B). Administration of 10⁻⁴ mol/L L-arginine to the bath solution did not significantly affect flow-induced constriction of HHcy arterioles (Figure 2C).

To reveal the possible source of superoxide, flow-induced responses were assessed after incubation of arterioles with the NAD(P)H-oxidase inhibitor DPI or the xanthine oxidase inhibitor oxypurinol. DPI had no effect on flow-induced responses (Figure 3A), whereas oxypurinol reversed flow-induced constriction of HHcy arterioles to dilation (maximum 21±3 μm, Figure 3B).
Effect and Possible Role of ONOO$^-$

Authentic ONOO$^-$ elicited significant constriction of arterioles of control rats (Figure 4A), which was inhibited by 10$^{-4}$ mol/L urate, confirming that urate acts as a potent ONOO$^-$ scavenger in biological systems. Constrictions to ONOO$^-$ were reversible after washout. Inhibition of TxA$_2$ receptors with SQ 29,548 abolished arteriolar constrictions to ONOO$^-$ (Figure 4B). Intraluminal administration of 10$^{-4}$ mol/L urate abolished flow-induced constrictions of HHcy arterioles (Figure 4B), but, unlike SOD, it did not restore dilations. Also, urate had no effect on flow-induced dilations of control arterioles (not shown). In the simultaneous presence of SOD and urate in the arteriolar lumen, increases of intraluminal flow elicited substantial dilations of HHcy arterioles (maximum 29±6 µm, Figure 4B).

Discussion

The new findings of this study are that (1) in arterioles of HHcy rats, intraluminal administration of SOD plus CAT converted flow-induced TxA$_2$-mediated constrictions to NO-mediated dilations but did not restore the dilator PG mediation and (2) both flow-induced constrictions of HHcy arterioles and constrictions of control arterioles to authentic ONOO$^-$ were prevented by urate, a known scavenger of ONOO$^-$, or a TxA$_2$ receptor antagonist. In the present study, we investigated the underlying mechanisms for the simultaneous lack of arteriolar NO mediation and enhanced TxA$_2$ production shown to result in constriction to increases in intraluminal flow in HHcy. First, we confirmed our previous findings that in control arterioles, increases in flow elicit substantial dilations (Figure 1A), whereas in arterioles isolated from HHcy rats, increases in intraluminal flow elicited significant constrictions (Figure 1A and 1B) due to release of TxA$_2$. The findings that in isolated arterioles of HHcy rats, additional extracellular eNOS sub-

Figure 1. A, Flow-induced changes in diameter of skeletal muscle arterioles of control and HHcy rats in the absence and presence of the TxA$_2$ receptor antagonist SQ 29,548. B, Original tracing (representative of 4 separate experiments) showing the effects of arachidonic acid (AA), SQ 29,548, and the Ca$^{2+}$ antagonist nimodipine (10$^{-6}$ mol/L) on diameter of an HHcy arteriole (passive diameter 155 µm). C, Flow-induced arteriolar responses in the absence and presence of the free radical scavengers SOD/CAT (intraluminally, B). Data are mean±SEM (n=7 to 30).

Figure 2. Flow-induced changes in diameter of skeletal muscle arterioles of HHcy rats before and after administration of SOD/CAT and the cyclooxygenase inhibitor indomethacin (INDO) or the TxA$_2$ receptor antagonist SQ 29,548 (A) or the NOS inhibitor L-NAME (B) or in the absence and presence of L-arginine (C). Data are mean±SEM (n=4 to 14).
strate l-arginine did not affect flow-induced constrictions (Figure 2C), but that scavenging of ROS with SOD plus CAT or SOD alone converted flow-induced constrictions to dilations (Figure 1B) suggest that in HHcy arterioles, bioavailability of l-arginine for eNOS is preserved and increases in flow elicit NO synthesis, but the elevated level of superoxide inactivates the released NO. Furthermore, the finding that in the presence of SOD plus CAT, the TxA2 receptor blocker SQ 29,548 had no effect on flow-induced dilation of HHcy arterioles (Figure 2A) suggests that in HHcy, the elevated level of superoxide not only inactivates NO but also promotes flow-induced synthesis of the constrictor PG TxA2.

It is likely that the antioxidant treatment restored primarily NO mediation of flow-induced dilation of HHcy arterioles, because this response was abolished by inhibition of NO synthesis (Figure 2B) but was not affected by indomethacin (Figure 2A). Recently, increased superoxide production was also demonstrated in aorta of mice with genetic HHcy. It is also likely that ROS-induced impairment of NO and PG synthesis contribute to the HHcy-induced endothelial dysfunction in humans, because it could be reversed by oral administration of antioxidants (eg, vitamin E, vitamin C). Interestingly, short-term administration of homocysteine also impaired agonist-induced endothelium-dependent relaxation of rabbit aortic rings, which can be prevented by superoxide scavengers. Because SOD alone was as effective as coadministration of SOD plus CAT, we aimed to elucidate the source of superoxide production in HHcy. On the basis of our findings, it seems that primarily xanthine oxidase (Figure 3B), rather than NAD(P)H oxidase (Figure 3A), is the major source of arteriolar superoxide production in HHcy. Similar upregulation of microvascular xanthine oxidase–dependent superoxide production has been found previously in other pathophysiological conditions, including hypertension; thus, one could speculate that increased xanthine oxidase activity contributes to the correlation among plasma homocysteine and uric acid concentrations and blood pressure in humans. In addition, studies on cell cultures suggest that homocysteine may also promote increased generation of ROS by downregulating antioxidant mechanisms, such as SOD. 

Figure 3. Flow-induced changes in diameter of skeletal muscle arterioles of HHcy rats in the absence and presence of the NAD(P)H oxidase inhibitor DPI (A) or the xanthine oxidase inhibitor oxypurinol in the absence and presence of L-NAME (B). Data are mean±SEM (n=6 to 7).

Figure 4. A, Responses of control arterioles to authentic ONOO− (x axis, nominal bath concentration of ONOO−) in the absence and presence of the TxA2 receptor antagonist SQ 29,548 or the ONOO− scavenger urate. B, Flow-induced responses of arterioles of HHcy rats before and after administration of urate or urate plus SOD. Data are mean±SEM (n=4 to 7). C, Proposed scheme for mechanisms by which reactive oxygen and nitrogen species convert flow-induced arteriolar dilation to constriction in HHcy: (1) increases in intraluminal flow activates eNOS to release NO and cyclooxygenase (COX) to produce PGH2; (2) there is an enhanced generation of superoxide primarily by xanthine oxidase (XO), which scavenges dilator NO by forming ONOO−; and (3) superoxide and/or ONOO− promotes the formation of TxA2 from PGH2 instead of dilator PGs, which then elicit flow-induced arteriolar constrictions.
as intracellular glutathione, glutathione peroxidase, and/or SOD,\textsuperscript{20,22} all of which participate in the elimination of oxygen-derived free radicals.

We hypothesized that superoxide interacts with NO and generates ONOO\textsuperscript{-},\textsuperscript{31,36} which may be responsible for flow-induced arteriolar constriction. The feasibility of this mechanism in HHcy is supported by studies on microvascular endothelial cells in culture showing inactivation of NO by superoxide and generation of ONOO\textsuperscript{-} induced by high concentrations of homocysteine.\textsuperscript{37} We found that ONOO\textsuperscript{-} is a potent vasoactive agent that elicits substantial TXA\textsubscript{2}-mediated constriction in skeletal muscle arterioles (Figure 4A), similar to its action in cerebral arteries.\textsuperscript{34} Direct measurements of ONOO\textsuperscript{-} metabolism have shown that ONOO\textsuperscript{-} (formed from xanthine-oxidase–derived superoxide and NO) can be effectively scavenged by urate in biological systems.\textsuperscript{31} The finding that urate prevented ONOO\textsuperscript{-}-induced arteriolar constrictions (Figure 4A) confirms that urate acts as a potent ONOO\textsuperscript{-} scavenger under the present experimental conditions as well. Because flow-induced arteriolar constriction in HHcy was abolished by intraluminal administration of the same concentration of the urate (Figure 4B) that prevented arteriolar constrictions to exogenous ONOO\textsuperscript{-} (Figure 4A), it is likely that ONOO\textsuperscript{-} generation is responsible for flow-induced arteriolar constriction in HHcy. In addition, when SOD was coadministered with urate into the arteriolar lumen, increases of intraluminal flow elicited substantial dilations of HHcy arterioles (Figure 4B). These findings suggest that urate does not act in a functionally measurable manner as a superoxide scavenger in isolated arterioles under the present experimental conditions.

Collectively, it seems that in HHcy, superoxide eliminates NO by forming ONOO\textsuperscript{-}, which then promotes the formation of TXA\textsubscript{2}.\textsuperscript{38} This mechanism of action is supported by studies showing that superoxide (generated by xanthine oxidase plus xanthine) in the presence of an NO donor, likely by producing ONOO\textsuperscript{-}, significantly increased TXA\textsubscript{2} synthesis and elicited substantial TXA\textsubscript{2}-mediated constriction in the rat coronary circulation.\textsuperscript{39} A likely mechanism by which ONOO\textsuperscript{-} may interfere with arachidonic acid metabolism is an inactivation of PGI\textsubscript{2} synthase,\textsuperscript{36,38} thus promoting synthesis of TXA\textsubscript{2}, as suggested by Figure 1B.

On the basis of our present and previous findings,\textsuperscript{8–10} we propose a model for describing the endothelial signaling mechanisms by which ROS convert flow-induced arteriolar dilation to constriction in HHcy (Figure 4C). Accordingly, in HHcy, (1) increases in intraluminal flow elicit the release of NO and PGH\textsubscript{2}; (2) NO is scavenged by superoxide generated primarily by xanthine oxidase to form ONOO\textsuperscript{-}; and (3) ONOO\textsuperscript{-}, most likely by inhibiting PG\textsubscript{I}\textsubscript{2} synthase, promotes the formation of TXA\textsubscript{2} from PGH\textsubscript{2} instead of dilator PG\textsubscript{Is},\textsuperscript{38} which then elicits flow-induced arteriolar constrictions. Production of PGH\textsubscript{2}/TXA\textsubscript{2} can be further amplified by the enhanced levels of arachidonic acid\textsuperscript{40,44} and may contribute to the increased urinary excretion of TXB\textsubscript{2}, the metabolite of TXA\textsubscript{2}, which has been observed in patients with HHcy.\textsuperscript{41,42}

Interestingly, simultaneous impairment of NO mediation of arteriolar responses and an enhanced synthesis of constrictor PG\textsubscript{Is} also has been shown in hypertension,\textsuperscript{43} diabetes mellitus,\textsuperscript{44} and heart failure,\textsuperscript{45} diseases that are also thought to be associated with increased formation of free radicals,\textsuperscript{17,44} suggesting a common mechanism of action of endothelial dysfunction, although the final outcome of the pathophysiological alterations may be different because of the presence of additional factors, such as elevated plasma levels of asymmetrical dimethylarginine, an endogenous inhibitor of NOS.\textsuperscript{16,46} Long-term ROS generation in the vessel wall may enhance lipid peroxidation\textsuperscript{27,48} and oxidative modification of LDL,\textsuperscript{49} promoting the development of atherothrombotic vascular diseases.\textsuperscript{50} It is likely, however, that changes in the vasoactive function of arteriolar endothelium represent early effects of HHcy and that they precede structural changes of vascular wall.\textsuperscript{51}

A proper balance between release of NO and dilator and constrictor PG\textsubscript{Is} is important for the prevention of vascular diseases. Thus, in HHcy, the impaired NO release and upregulated synthesis of TXA\textsubscript{2} in the endothelium, together with the simultaneously increased TXA\textsubscript{2} synthesis in platelets,\textsuperscript{9,40} can promote platelet aggregation, favor thrombus formation, and may lead to occlusive peripheral vascular disease, such as intermittent claudication, in addition to atherosclerotic alterations of larger arteries.\textsuperscript{17,44}

In conclusion, the present study is the first to demonstrate that the flow-dependent arteriolar dilation observed in normal healthy conditions is converted to constriction in hyperhomocysteinemic rats because of an enhanced production of superoxide primarily by xanthine oxidase, which decreases the bioavailability of NO, most likely through the formation of ONOO\textsuperscript{-}, and promotes the enhanced release of TXA\textsubscript{2}. Such alterations in the vasoactive function of endothelium in hyperhomocysteinemia could lead to atherothrombotic diseases and limit or reduce tissue perfusion, thereby aggravating symptomatic peripheral arterial disease.

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