Impaired Nitric Oxide-Mediated Flow-Induced Coronary Dilation in Hyperhomocysteinemia

Morphological and Functional Evidence for Increased Peroxynitrite Formation

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Hyperhomocysteinemia (HHcy) is a newly recognized risk factor for myocardial infarction, however, the effect of HHcy on endothelium-dependent flow-induced dilation of coronary arteries is not known. Thus, changes in diameter of small intramural coronary arteries (diameter, $\sim 145 \mu\text{m}$) isolated from control rats and rats with methionine diet-induced HHcy were investigated by videomicroscopy. Increases in intraluminal flow (from 0 to $40 \mu\text{l/min}$) elicited dilations of control vessels (maximum, $25 \pm 2 \mu\text{m}$), responses that were absent in HHcy arteries. The nitric oxide (NO) synthase inhibitor L-NAME inhibited flow-induced dilation of control coronaries, whereas it had no effect on responses of HHcy arteries. Dilations of control and HHcy arteries to the NO donor sodium nitroprusside were not different. Responses to flow in HHcy coronary arteries were unaffected by administration of L-arginine or the prostaglandin H_2 /thromboxane A_2 receptor antagonist SQ 29,548. However, in the presence of superoxide dismutase (plus catalase) or the superoxide scavenger Tiron increases in flow elicited L-NAME-sensitive dilations of HHcy coronaries (maximum, $18 \pm 5 \mu\text{m}$). Also, superoxide dismutase significantly reduced the enhanced superoxide production of HHcy coronaries (measured by the lucigenin chemiluminescence method). Single vessel Western blotting showed an increased tyrosine nitrosation (a stable biomarker of tissue peroxynitrite formation) in HHcy coronaries. Also, extensive prevalence of 3-nitrotyrosine immunoreactivity was observed in HHcy coronaries that was confined primarily to the subendothelial layers of smooth muscle. We propose that in HHcy an increased level of superoxide scavenges NO forming peroxynitrite, which increases protein nitrosation. The reduced bioavailability of NO impairs flow-induced dilations of coronary arteries, which may contribute to the development of

coronary atherosclerosis and ischemic heart disease. (*Am J Pathol* 2002, 161:145–153)

Homocysteine is a thiol-containing amino acid that is formed from methionine, an essential amino acid found in large quantities in meat. Increased plasma concentration of homocysteine (hyperhomocysteinemia, HHcy) is an independent risk factor for occlusive peripheral artery disease, ischemic heart disease, and myocardial infarction.^{1–4}

One of the mechanisms by which HHcy elicits its adverse vascular effects is by altering endothelial function. Recent studies have shown that even mild HHcy (15 to $30 \mu\text{mol/L}$) is associated with significant impairment of endothelium-dependent vasoactive responses in large arteries and aorta of monkeys⁵ and mice,⁶ as well as in rat skeletal muscle arterioles.^{7–9} Despite this compelling evidence, there is little data regarding HHcy-induced alterations in the coronary circulation, where potential HHcy-induced pathology could contribute greatly to fatal cardiac events.

Flow-induced dilation of small coronary vessels is one of the most important mechanisms contributing to the local regulation of myocardial blood flow, is a response that is mediated by endothelium-derived nitric oxide (NO) in most species.^{10,11} Because NO exerts both vasodilatory and anti-aggregatory actions, the impairment of flow-induced NO release likely leads to ischemic episodes and contributes to the development of coronary heart disease. Indeed, it has been shown that pathological conditions that are associated with an increased risk of coronary heart disease, such as arteriosclerosis, diabetes, and hypertension are characterized by a decreased

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NO synthesis/release interfering with endothelium-dependent regulation of coronary circulation.¹²

HHcy may affect endothelial function of coronary vessels via mechanisms that increase oxidative stress, as suggested by recent studies on endothelial cells in culture.^{13,14} Indeed, previous studies have demonstrated that enhanced oxidative stress impairs endothelial functions in hypercholesterolemia, diabetes, and hypertension.¹⁵ One of the key reactive oxygen metabolites is superoxide (O_2^-), which interacts with NO and generates peroxynitrite ($ONOO^-$).¹⁶ It could be hypothesized that NO-mediated flow-induced coronary dilation is impaired because of an enhanced generation of superoxide in HHcy, which scavenges NO (released to increases in flow) forming $ONOO^-$. This mechanism may contribute to the development of coronary heart disease in HHcy.

To test this hypothetical mechanism we investigated flow-induced responses in isolated small intramural coronary arteries of HHcy rats in the presence and absence of pharmacological probes of known action on synthesis of endothelial factors and metabolism of superoxide. Furthermore, increased *in vivo* generation of $ONOO^-$ in HHcy coronary arteries was evidenced by detecting protein tyrosine nitrosation (a stable biomarker of tissue $ONOO^-$ formation)^{16–19} with single vessel Western blotting and immunohistochemistry.

Materials and Methods

Male Wistar rats ($n = 46$; 300 to 330 g, Charles River Co., Wilmington, MA) were housed separately and had free access to water and standard rat chow. In one group ($n = 23$), moderate HHcy was induced by administration of L-methionine (0.5 g/kg body weight/day) in the drinking water for a period of 4 weeks. Previous studies and our present findings show that this methionine-rich diet increases plasma homocysteine concentration threefold to fivefold in rats.^{7,8} Plasma homocysteine and uric acid concentrations were measured with commercially available kits (Abbott Diagnostics Co., Sigma Co., St. Louis, MO).

Isolation of Intramural Coronary Arteries and Videomicroscopy

Experiments were conducted on isolated small intramural coronary arteries (diameter, $\sim 145 \mu m$), as described previously.^{7–9,12} In brief, the heart was excised, the septum was exposed, and a branch ~ 1.5 mm in length of the septal coronary artery running intramuscularly was isolated. The arteriole was cannulated on both sides in an organ chamber containing physiological salt solution (in mmol/L: 110 NaCl, 5.0 KCl, 2.5 $CaCl_2$, 1.0 $MgSO_4$, 1.0 KH_2PO_4 , 5.0 glucose, and 24.0 $NaHCO_3$, equilibrated with 10% O_2 , 5% CO_2 , 85% N_2 , at pH 7.4, 37°C). Pressure on both sides was adjusted by a pressure servo-control system. Internal diameter at the midpoint of the arterial segment was measured with videomicroscopy. 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 intraluminal pressure constant⁹ and was measured with a ball flowmeter (Omega Engineering Inc.).

Functional Studies

After the equilibration period changes in diameter of isolated coronaries were assessed in response to step increases in intraluminal flow (from 0 to 50 $\mu l/min$). Then the vessels were exposed in various protocols to incubation with N^ω -nitro-L-arginine-methyl-ester (L-NAME) (3×10^{-4} mol/L, eNOS inhibitor) or extracellular L-arginine (10^{-4} mol/L, the substrate of eNOS) or SQ 29,548 (10^{-6} mol/L for 20 minutes, a TxA_2 /PGH₂ receptor inhibitor), or the free radical scavengers Tiron (4,5-dihydroxy-1,3-benzene-disulphonic acid, 10 mmol/L, a spin trap known to eliminate superoxide in coronary endothelium²⁰), or superoxide dismutase (SOD) (120 U/ml for 30 minutes, a potent scavenger of superoxide in coronary arteries²⁰ and isolated arterioles²¹) plus catalase (CAT) (80 U/ml, for 30 minutes, to eliminate H_2O_2 formed by SOD)²¹ or SOD plus L-NAME or the $ONOO^-$ scavenger urate¹⁶ (10^{-4} mol/L for 30 minutes) and flow-induced responses were obtained again. Flow (40 $\mu l/min$)-induced responses of HHcy arteries were also assessed before and after administration of aminoguanidine (10^{-3} mol/L, an iNOS inhibitor), indomethacin (3×10^{-5} mol/L, a COX inhibitor), diphenyliodonium [10^{-4} mol/L, abluminally, an inhibitor of flavin-containing enzymes, including NAD(P)H oxidases] or dithiothreitol (10^{-3} mol/L, a thiol-reducing agent thought to reverse $ONOO^-$ -induced cysteine oxidation²²). Arteriolar responses to the NO donor sodium nitroprusside (10^{-9} to 10^{-6} mol/L) were also assessed. In separate experiments responses of control arterioles to increasing concentrations of authentic $ONOO^-$ were also obtained in the presence and absence of uric acid. In other experiments flow-induced responses of control arteries were obtained, then the vessels were incubated with authentic $ONOO^-$ (nominal final concentration, 10 $\mu mol/L$) for 20 minutes followed by a 10-minute washout and flow-induced responses were obtained again.

At the conclusion of each experiment to obtain the maximum passive diameter, the suffusion solution was changed to a Ca^{2+} -free solution, which contained EGTA (ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid, 10^{-3} mol/L), the vessel was incubated for 10 minutes and changes in passive arteriolar diameter in response to changes in intraluminal pressure (20 to 140 mmHg) were obtained.

Measurement of Vascular Superoxide Level

Vascular O_2^- production was assessed by the lucigenin chemiluminescence method⁶ in collaboration with Dr. Pavel Kaminski and Dr. Michael S. Wolin according to the modified protocol of Mohazzab and colleagues.²³ In brief, coronary arteries were isolated from control ($n = 8$) and HHcy ($n = 8$) rats. Vessels from each animal were pooled and placed in scintillation vials containing 1 ml of

physiological buffer solution and 10 $\mu\text{mol/L}$ of lucigenin. Lucigenin chemiluminescence was measured in a liquid scintillation counter (Mark V; TmAnalytic, Elk Grove Village, IL) with a single active photomultiplier tube positioned in out-of-coincidence mode in the darkroom. Scintillation counts were obtained 15 to 20 minutes after addition of vessels (averaged) in the absence or presence of SOD and background-corrected values were normalized to tissue weight. In separate experiments O₂⁻ production by control coronary arteries and vessels preincubated with authentic ONOO⁻ (nominal final concentration, 10 $\mu\text{mol/L}$ for 20 minutes) were compared.

Materials

Authentic ONOO⁻ was obtained from Calbiochem (La Jolla, CA) as an aqueous stock solution in 4.7% NaOH (nominal concentration, $\sim 200 \text{ mmol/L}$) and was stored aliquoted at -80°C under inert gas, and protected from light throughout the experiments. Immediately before each experiment, an aliquot of the stock solution was diluted into an ice-cold alkaline solution²⁴ from which an appropriate volume was administered immediately in the organ bath. In Figure 2 the amount of ONOO⁻ administered to a 10-ml bath solution is expressed. SQ 29,548 (Cayman Chemicals, Ann Arbor, MI) was dissolved in ethanol and urate (Calbiochem) was dissolved in alkaline buffer. All other salts and chemicals were obtained from Sigma-Aldrich Co. and solutions were prepared on the day of the experiment. All drugs were added to the vessel chamber, and final concentrations are reported.

Detection of Coronary 3-Nitrotyrosine Content with Western Blotting

Single small coronary arteries from the same control and HHcy rats that were used for functional studies were isolated, snap-frozen in liquid nitrogen, and stored at -80°C . Isolated coronary arteries of control rats incubated with authentic ONOO⁻ (nominal concentration, 10 $\mu\text{mol/L}$) served as positive controls. Samples were homogenized by sonification in Laemmli buffer, boiled for 5 minutes, and loaded onto a 10% acrylamide gel (Criterion Gels; Bio-Rad, Richmond, CA), resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to Hybond P (Amersham Life Science, Arlington Heights, IL) membrane at 1 mA/cm² for 60 minutes with a semi-dry blotting system (Bio-Rad). The membranes were blocked in Tris-buffered saline-Tween 20 (TBST) buffer containing 5% nonfat milk for overnight at 4°C. Primary polyclonal anti-3-nitrotyrosine antibody (1:750; Cell Signaling Technology Co., Beverly, MA) or monoclonal anti-GAPDH antibody (1:2500; Chemicon Co.) was added to the membrane for 1 hour at room temperature. The membranes were washed with phosphate-buffered saline (PBS) and incubated for 1 hour with sheep anti-rabbit IgG-horseradish peroxidase or donkey anti-mouse IgG-horseradish peroxidase (Amersham) at the final titer of 1:4000. The membranes were developed with ECL-Plus (Amersham) and analyzed with densitom-

etry. Anti-GAPDH was used to normalize for loading variations. In separate bioassay experiments arteries of control rats were incubated with ONOO⁻ in the absence and presence of urate (10^{-4} mol/L) and 3-nitrotyrosine content was detected.

Immunohistochemical Detection of 3-Nitrotyrosine

Tissue samples from the interventricular septum were embedded in OTC 4583 medium (Sakura Finetek, Torrance, CA), snap-frozen in liquid nitrogen, and immunolabeling was performed according to the modified protocol of Baker and colleagues¹⁹ and others.^{17,18,25} Tissue sections (thickness, 4 μm) were cut by using a cryostat and collected on Probe-on-Plus (Fisher Scientific, Pittsburgh, PA) microscope slides. Sections were fixed with cold (4°C) acetone and exposed to 3% hydrogen peroxide (5 minutes, 25°C, in methanol) to quench endogenous peroxidase activity. Slides were washed three times for 5 minutes with PBS. To block nonspecific binding of antibodies, sections were incubated for 4 hours (25°C) with 2.5% normal horse serum, which was then aspirated from each section. Then sections were incubated overnight at 4°C with optimally diluted (in PBS containing 0.1% bovine serum albumin and 0.2% Triton X-100) primary antibody against nitrated tyrosine residues of proteins (1:100; Cell Signaling Technology Co.). To label bound primary antibody, sections were incubated for 45 minutes at 25°C with a 1:100 dilution of goat anti-rabbit secondary antibody conjugated to biotin (Vector Laboratories, Burlingame, CA). Between each step of labeling protocol sections were rinsed in 0.1% Triton X-100 in 0.05 mol/L of Tris-saline solution. Sections were then incubated with avidin-biotinylated enzyme complex (ABC Vectastain, Vector Laboratories), stained with Vector-DAB (diaminobenzidine tetrahydrochloride) substrate and counterstained with hematoxylin. Some samples were stained with Vector-VIP substrate to increase specificity and counterstained with methyl green. Sections were then rinsed in distilled water, permanently mounted with VectaMount medium, and covered with a coverslip. The specificity of the immunolabeling was evaluated by omitting the primary antibody or omitting both primary and secondary antibodies in control experiments. Further specificity controls were made by immunostaining sections after overnight incubation of the primary antibody with the homologous antigen for 3-nitrotyrosine (10 mmol/L). Images of the sections were collected with a Cool-Snap-CF CCD camera (Roper Scientific, Tucson, AZ) connected to an Olympus BX60 microscope.

Data Analysis

Dilation of isolated arteries were expressed as absolute values. Statistical analyses were performed by two-way analysis of variance for repeated measures followed by the Tukey post hoc test or Student's *t*-test, as appropriate. $P < 0.05$ was considered statistically significant. Data are expressed as means $\pm$ SEM.

Results

There was no significant difference between body weight (control, 330 ± 15 g; HHcy, 319 ± 20 g) of control and methionine-fed rats. Serum homocysteine concentrations in control and HHcy rats were 5.9 ± 0.6 $\mu\text{mol/L}$ and 29.9 ± 6.5 $\mu\text{mol/L}$ ($P < 0.01$), respectively, whereas serum uric acid concentrations were $2.54 \pm 0.07 \times 10^{-4}$ mol/L (control) and $2.38 \pm 0.05 \times 10^{-4}$ mol/L (HHcy, n.s.). Isolated coronary arteries of control and HHcy rats developed active tone in response to intraluminal pressure of 80 mmHg without the use of any vasoactive agent (control, 140 ± 8 μm ; HHcy, 149 ± 10 μm , n.s.). The passive diameters of control and HHcy coronaries in the absence of extracellular Ca^{2+} were 223 ± 11 μm and 227 ± 13 μm (n.s.), respectively (at 80 mmHg).

Flow- and Agonist-Induced Dilations of Coronary Arteries

Stepwise increases in intraluminal flow elicited significant dilations in isolated coronary arteries of control rats, responses that were inhibited by L-NAME (Figure 1, A and B). In contrast, in coronary arteries of HHcy rats flow-induced responses were essentially absent both in the presence and absence of L-NAME (Figure 1, A and B). Dilations to cumulative doses of sodium nitroprusside were not significantly different between control and HHcy arteries (Figure 1C). Flow-induced responses of HHcy arteries were not affected by the presence of excess extracellular L-arginine (10^{-4} mol/L) or by administration of the $\text{PGH}_2/\text{TxA}_2$ receptor antagonist SQ 29,548 (10^{-6} mol/L, Figure 2C).

To assess the role of reactive oxygen species in impaired flow-induced responses of HHcy arteries we administered exogenous SOD (a method that was shown to effectively scavenge superoxide in coronary arteries²⁰ and isolated arterioles²¹) plus catalase, Tiron, or urate. SOD (plus catalase) restored flow-induced dilations of HHcy arteries (Figure 2B), but did not affect flow-induced dilations of control arteries (Figure 2A). In the presence of SOD (plus catalase) flow-induced dilations of HHcy arteries were abolished by L-NAME (maximum, 5 ± 3 μm). The structurally different superoxide scavenger Tiron also restored flow-induced dilations of HHcy arteries (Figure 2C) that were inhibited by L-NAME (maximum, 6 ± 4 μm). Uric acid, a potent ONOO^- scavenger,¹⁶ had no effect on flow-induced responses of HHcy arteries (Figure 2C). Flow-induced responses of HHcy arteries were augmented by abluminal application of diphenyliodonium, but were unaffected by aminoguanidine, indomethacin, or dithiothreitol (Figure 2D). Administration of dithiothreitol elicited an ~ 10 - μm dilation that was followed by a gradual ~ 15 - μm constriction.

Vasoactive Effects of ONOO^-

Low doses of authentic ONOO^- (10^{-8} to 10^{-4} mmol ONOO^- administered to 10 ml bath solution; corresponding to calculated nominal final concentrations: 10^{-9} to

Figure 1. Flow-induced changes in the diameter of intramural coronary arteries of control and hyperhomocysteinemic (HHcy) rats in the absence (**A**) and presence (**B**) of the NO synthase inhibitor L-NAME. **C:** Coronary dilations to sodium nitroprusside, a NO donor. Data are mean $\pm$ SEM ($n = 7$ to 15). *, $P < 0.05$.

10^{-5} mol/L, although actual effective concentrations are likely to be lower because of the short half-life of ONOO^- at physiological pH) did not have significant vasoactive effects, whereas ONOO^- at high doses elicited slight, but significant, constrictions of coronary arteries of control rats that were completely abolished by uric acid (Figure 2E). Decomposed ONOO^- (ONOO^- was incubated for 30 minutes in Krebs' solution at 37°C), unlike acute administration of authentic ONOO^- , did not elicit

Figure 2. Flow-induced changes in diameter of intramural coronary arteries of control (**A**) and hyperhomocysteinemic (HHcy, **B**) rats before and after administration of SOD plus catalase (SOD/CAT). **C:** Flow-induced responses of HHcy coronaries after administration of the free radical scavenger Tiron (10 mmol/L) or L-arginine (10^{-4} mol/L) or uric acid (10^{-4} mol/L) or the TxA₂ receptor inhibitor SQ 29,548 (10^{-6} mol/L). **D:** Flow (40 μL/min)-induced responses of HHcy vessels in the absence and presence of diphenyliodonium (10^{-4} mol/L) or aminoguanidine (10^{-4} mol/L) or indomethacin (3×10^{-5} mol/L) or dithiothreitol (10^{-3} mol/L). **E:** Responses of control arteries to authentic ONOO⁻ (amount of ONOO⁻ administered to 10 ml of bath solution is expressed). **F:** Flow (40 μL/minute)-induced responses of control vessels before and after incubation with ONOO⁻ (10 μmol/L, for 20 minutes, followed by 10 minutes of washout). Data are mean $\pm$ SEM ($n = 4$ to 7). *, $P < 0.05$.

vasoactive effects (not shown). Flow-induced dilations of control coronary arteries were not affected significantly by pretreatment with ONOO⁻ (Figure 2F).

Vascular Superoxide Level in Coronary Arteries

Under baseline conditions, levels of O₂⁻ produced by coronary arteries of HHcy rats were significantly higher (by ~4-fold) than in vessels of control rats (as detected by lucigenin-enhanced chemiluminescence; Table 1). Treatment with SOD significantly decreased superoxide levels in control and HHcy arteries eliminating the difference between the groups (Table 1). Preincubation of

Table 1. Superoxide Production in Coronary Arteries from Control and HHcy Rats

	Chemiluminescence (10^3 cpm/mg)	
	Untreated	SOD
Control	12.7 $\pm$ 1.6	4.9 $\pm$ 1.0 [†]
HHcy	51.53 $\pm$ 12.0*	13.3 $\pm$ 6.8 [†]

Vascular O₂⁻ production assessed by lucigenin (10 μmol/L) chemiluminescence in isolated coronary arteries from control ($n = 8$) and HHcy ($n = 8$) rats in the absence or presence of SOD.

* Significant ($P < 0.05$) difference between groups.

† Significant difference between vessels with or without treatment.

Data are expressed as percentage of control mean $\pm$ SEM.

control coronary arteries with authentic ONOO⁻ had no significant effect on vascular O₂⁻ production (control, $100 \pm 25\%$; control + ONOO⁻, $128 \pm 23\%$; $n = 4$ in each group, data are normalized to control mean).

Detection of Coronary 3-Nitrotyrosine Content with Western Blotting

Western blot analysis of coronary artery lysates showed an extensive prevalence of nitrated tyrosine residues of proteins in HHcy coronaries, whereas 3-nitrotyrosine immunoreactivity was weak in control vessels (a Western blot representative to three separate experiments is shown in Figure 3A). In control arteries pretreated with authentic ONOO⁻ (Figure 3A, lanes 1 and 2) there was a strong 3-nitrotyrosine immunoreactivity that had a similar pattern as the 3-nitrotyrosine immunoreactivity found in HHcy coronaries. Figure 3B shows the summary data of the total background-corrected band densities normalized to the arterial GAPDH content indicating that 3-nitrotyrosine content of HHcy coronaries was significantly increased, as compared to vessels of control rats (Figure 3B). In separate experiments we showed that acute formation of 3-nitrotyrosine by pretreatment of control arteries with ONOO⁻ can be prevented by the ONOO⁻ scavenger urate (Figure 3C). Administration of decomposed ONOO⁻ did not result in detectable 3-nitrotyrosine immunoreactivity.

Immunohistochemical Detection of 3-Nitrotyrosine

Samples from the hearts of four HHcy animals were analyzed with immunohistochemistry. In large intramural coronary arteries of HHcy rats immunostaining for nitrotyrosine was localized mainly to the subendothelial layers of the media (Figure 4A), whereas the endothelium was relatively free from immunoreactivity (inset, arrow). Similar subendothelial localization of 3-nitrotyrosine immunoreactivity was found in large (Figure 4C) and small intramural coronary arteries and arterioles (Figure 4D), whereas the endothelium of these vessels was also relatively free from 3-nitrotyrosine immunoreactivity (Figure 4D). Immunostaining for 3-nitrotyrosine also could not be detected in the myocardium. In control experiments on consecutive tissue sections in the absence of the primary antibody or both primary and secondary antibodies or after absorption of the primary antibody with the homologous antigen for 3-nitrotyrosine (Figure 4, B and F) there was no evidence for nonspecific immunostaining.

Discussion

The new findings of this study are that in rat HHcy 1) flow-induced NO-mediated dilation of isolated intramural small coronary arteries is abolished; 2) it can be restored by administration of superoxide scavengers SOD or Tiron; 3) superoxide production by coronary arteries is significantly increased and can be inhibited by exoge-

Figure 3. **A:** Representative Western blot showing protein tyrosine nitrosation in small intramural coronary arteries of hyperhomocysteinemic (HHcy, lanes 3 and 4) and control (lanes 5 and 6) rats. Coronary arteries of control rats incubated with authentic ONOO⁻ (10 μ mol/L) served as positive controls (lanes 1 and 2). Anti-GAPDH was used to normalize for loading variations. **B:** Summary data for densitometric analysis of protein tyrosine nitrosation in single control ($n = 8$) and HHcy ($n = 8$) coronary arteries. *, $P < 0.05$. **C:** Representative Western blot showing protein tyrosine nitrosation in arteries incubated with ONOO⁻ in the absence and presence of urate (10^{-4} mol/L), a scavenger of ONOO⁻.

nous SOD; 4) the vascular protein 3-nitrotyrosine content, which is predominantly confined to the subendothelial smooth muscle of the coronary arterial wall, is significantly increased.

To elucidate the effect of HHcy on endothelial regulation of coronary resistance vessels first we demonstrated that under control conditions increases in flow elicit substantial dilations of small coronary arteries, a response that primarily depends on the endothelial release of NO (Figure 1).^{10,11} In contrast, flow-induced responses were essentially eliminated in coronary arteries isolated from rats with diet-induced chronic (4 weeks) HHcy (Figure 1). The finding that neither L-NAME nor the TxA₂ receptor inhibitor SQ 29,548 affected the lack of dilation of HHcy coronaries suggest that it is most likely because of an impaired NO mediation⁹ (Figures 1 and 2). These findings have important clinical applications, because in humans a similar degree of mild HHcy, either chronic or transient after an experimental methionine load, is also associated with an impaired dilation of peripheral arteries in response to infusion of ACh and/or to a release of an occlusion.^{26–28} Thus, it is likely that HHcy impairs NO mediation of flow-induced dilation and thereby limits myocardial blood flow and promotes platelet aggregation and/or imposes greater demand on other compensatory mechanisms and may lead to ischemic heart disease. Because dilation of HHcy coronary arteries to the NO donor sodium nitroprusside were normal (Figure 1C), it is likely that the dilator capacity of the smooth muscle is unaffected by 4-week HHcy.

The finding, that scavenging of reactive oxygen species with SOD, which had no significant effect on responses of control coronaries (Figure 2A), restored flow-induced dilation in arteries of HHcy rats (Figure 2B) suggests that in HHcy arteries increases in flow do elicit NO synthesis, but an increased production of reactive oxygen species, predominantly superoxide, inactivates the released NO. This idea is further supported by the findings that Tiron, a structurally different cell-permeable superoxide scavenger also effectively restored flow-induced dilations of HHcy coronary arteries (Figure 2C). An increased superoxide production in HHcy coronaries was also evidenced by the significantly increased lucigenin chemiluminescence in these vessels (Table 1), corresponding well with similar findings in the aorta of mice with genetic HHcy suggesting that increased superoxide production is independent from the model of HHcy.⁶ Because in the present study the increased lucigenin chemiluminescence was substantially inhibited by SOD (Table 1) it is likely that HHcy is primarily associated with an increased extracellular production of superoxide in the coronary arteries. An important *in vivo* role for increased superoxide production in HHcy is supported by observations that endothelial function in humans with high homocysteine plasma concentrations can be improved by oral administration of antioxidants (eg, vitamin E, vitamin C).^{29,30}

Because flow-induced responses of HHcy coronary arteries were significantly augmented by diphenyliodonium, it is likely that an increased activity of vascular NAD(P)H oxidase,²³ rather than iNOS³¹ or cyclooxygen-

Figure 4. In coronary arteries of hyperhomocysteinemic rats, immunostaining for nitrotyrosine was localized mainly to the subendothelial layers of the media (**A**, **arrowhead**, brown reaction product, diaminobenzidine staining with hematoxylin counterstaining) and the endothelium was relatively free from immunoreactivity (**inset**, **arrow**). **B**: Consecutive sections served as negative control (antigen absorption). Similar subendothelial localization of 3-nitrotyrosine immunoreactivity was found in large (**C**) and small intramural coronary arteries and arterioles (**D**, red product, **arrowheads**, VIP staining with methyl green counterstaining). The endothelium of small coronary arteries was relatively free from 3-nitrotyrosine immunoreactivity (**E**, brown product, DAB, longitudinal section; **F**, consecutive negative control). Scale bars, 50 μm (**A–C**); 25 μm (**D–F**).

ase,⁸ contributes to superoxide production in HHcy coronary arteries (Figure 2D). In addition, HHcy may also alter cellular redox state by activating xanthine oxidase³² and/or down-regulating the expression of glutathione peroxidase.³³ Because administration of L-arginine did not restore flow-induced dilations in HHcy coronaries

(Figure 2C), it is unlikely that HHcy-induced endothelial dysfunction is due to reduced L-arginine availability.

It is known that interaction of superoxide with NO *in vitro* results in the formation of ONOO^- .^{16,34} Because single vessel Western blotting revealed that in coronary arteries of HHcy rats there is an increased protein 3-ni-

trotyrosine content (a stable reaction product of ONOO⁻;^{16–19} Figure 3), it is likely that in chronic HHcy an increased level of superoxide scavenges NO released to the constant presence of coronary blood flow resulting in increased formation of ONOO⁻ *in vivo*. Further evidence for ONOO⁻ formation in HHcy is also provided by studies showing 3-nitrotyrosine staining in the aorta of mice with genetic HHcy⁶ and by studies on endothelial cells in culture showing inactivation of NO by superoxide and generation of ONOO⁻ induced by high concentrations of homocysteine.³⁵ In the present study we found that protein tyrosine nitrosation in HHcy arteries is predominantly confined to the subendothelial smooth muscle layer of the arterial wall (as shown with immunohistochemistry, Figure 3), raising the possibility that smooth muscle cells in this region are significant sources for production of superoxide and ONOO⁻. Indeed, previous studies showed that NAD(P)H oxidase expressed in the smooth muscle cells is a major source of O₂⁻.^{23,36,37} It is of note that a similar pattern of immunostaining for 3-nitrotyrosine was observed both in large and small coronary arteries and arterioles (Figure 4; D and E), suggesting that adverse effects of HHcy, similar to hypercholesterolemia,¹² extend into the coronary microcirculation.

The pathological role of ONOO⁻ is likely to be multifaceted. Although acute administration of high doses of exogenous ONOO⁻ may elicit vasoconstriction *in vitro*^{24,38} (Figure 2E) flow-induced responses were not affected by the potent ONOO⁻ scavenger uric acid¹⁶ in coronary arteries of HHcy rats (Figure 2C and Figure 3C) suggesting that ONOO⁻ does not act directly as a vasoactive mediator in coronary arteries in HHcy. It is also unlikely that oxidation of protein cysteine residues by ONOO⁻²² impairs NO mediation in HHcy, because flow-induced responses of HHcy arteries were unaffected by dithiothreitol (Figure 2D). The hypothesis that protein nitrosation by ONOO⁻ inactivates eNOS in HHcy could also be refuted because *in vitro* incubation of control vessels with authentic ONOO⁻, which results in substantial nitrosation of vascular proteins (Figure 3), did not affect flow-induced responses (Figure 2F). Furthermore, flow-induced dilations of HHcy coronary arteries were reversible by acute administration of superoxide scavengers (Figure 2, B and C). Nevertheless, our data do not rule out that chronic superoxide/ONOO⁻-induced protein/lipoprotein modification (either tyrosine nitrosation or cysteine oxidation) affects other functions of coronary arteries and/or contribute to the development of arteriosclerosis.^{17,39,40} ONOO⁻ may also elicit DNA single-strand breaks activating the poly(ADP-ribose) polymerase³² predisposing coronary vessels, especially when other risk factors are present, for pathophysiological alterations.²⁵ Also, HHcy may trigger other cellular mechanisms (eg, activation of redox-sensitive genetic elements, production of cytokines,⁴¹ down-regulation of glutathione peroxidase³³) that can further aggravate vascular dysfunction and lead to arteriosclerotic alterations, isoprostane formation,⁶ lipid peroxidation,⁴² and smooth muscle proliferation.⁴³

In conclusion, the present study demonstrated that flow-induced dilation of isolated small intramural coro-

nary arteries is substantially impaired in hyperhomocysteinemic rats because of an enhanced production of superoxide, which decreases the bioavailability of NO to mediate the dilation through the formation of ONOO⁻. Such alterations in endothelial regulation of coronary circulation in HHcy could promote the development of arteriosclerosis and ischemic heart disease.

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References

1. Nygard O, Nordrehaug JE, Refsum H, Ueland PM, Farstad M, Vollset SE: Plasma homocysteine levels and mortality in patients with coronary artery disease. *N Engl J Med* 1997, 337:230–236
2. Chambers JC, Obeid OA, Refsum H, Ueland P, Hackett D, Hooper J, Turner RM, Thompson SG, Kooner JS: Plasma homocysteine concentrations and risk of coronary heart disease in UK Indian Asian and European men. *Lancet* 2000, 355:523–527
3. Foody JM, Milberg JA, Robinson K, Pearce GL, Jacobsen DW, Sprecher DL: Homocysteine and lipoprotein(a) interact to increase CAD risk in young men and women. *Arterioscler Thromb Vasc Biol* 2000, 20:493–499
4. Stampfer MJ, Malinow MR, Willett WC, Newcomer LM, Upson B, Ullmann D, Tishler PV, Hennekens CH: A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. *JAMA* 1992, 268:877–881
5. Lentz SR, Sobey CG, Piegors DJ, Bhopatkar MY, Faraci FM, Malinow MR, Heistad DD: Vascular dysfunction in monkeys with diet-induced hyperhomocyst(e)inemia. *J Clin Invest* 1996, 98:24–29
6. Eberhardt RT, Forgione MA, Cap A, Leopold JA, Rudd MA, Trolliet M, Heydrick S, Stark R, Klings ES, Moldovan NI, Yaghoubi M, Goldschmidt-Clermont PJ, Farber HW, Cohen R, Loscalzo J: Endothelial dysfunction in a murine model of mild hyperhomocyst(e)inemia. *J Clin Invest* 2000, 106:483–491
7. Ungvari Z, Pacher P, Rischak K, Szollar L, Koller A: Dysfunction of nitric oxide mediation in isolated rat arterioles with methionine diet-induced hyperhomocysteinemia. *Arterioscler Thromb Vasc Biol* 1999, 19:1899–1904
8. Ungvari Z, Sarkadi-Nagy E, Bagi Z, Szollar L, Koller A: Simultaneously increased TxA₂ activity in isolated arterioles and platelets of rats with hyperhomocysteinemia. *Arterioscler Thromb Vasc Biol* 2000, 20:1203–1208
9. Bagi Z, Ungvari Z, Szollar L, Koller A: Flow-induced constriction in arterioles of hyperhomocysteinemic rats is due to impaired nitric oxide and enhanced thromboxane A(2) mediation. *Arterioscler Thromb Vasc Biol* 2001, 21:233–237
10. Muller JM, Davis MJ, Kuo L, Chilian WM: Changes in coronary endothelial cell Ca²⁺ concentration during shear stress- and agonist-induced vasodilation. *Am J Physiol* 1999, 276:H1706–H1714
11. Kuo L, Davis MJ, Chilian WM: Endothelium-dependent, flow-induced dilation of isolated coronary arterioles. *Am J Physiol* 1990, 259: H1063–H1070
12. Kuo L, Davis MJ, Cannon MS, Chilian WM: Pathophysiological consequences of atherosclerosis extend into the coronary microcirculation. Restoration of endothelium-dependent responses by L-arginine. *Circ Res* 1992, 70:465–476
13. Upchurch Jr GR, Welch GN, Fabian AJ, Freedman JE, Johnson JL, Keaney Jr JF, Loscalzo J: Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. *J Biol Chem* 1997, 272:17012–17017
14. Lang D, Kredan MB, Moat SJ, Hussain SA, Powell CA, Bellamy MF, Powers HJ, Lewis MJ: Homocysteine-induced inhibition of endothe-

- lium-dependent relaxation in rabbit aorta: role for superoxide anions. *Arterioscler Thromb Vasc Biol* 2000, 20:422–427
15. Suzuki H, DeLano FA, Parks DA, Jamshidi N, Granger DN, Ishii H, Suematsu M, Zweifach BW, Schmid-Schonbein GW: Xanthine oxidase activity associated with arterial blood pressure in spontaneously hypertensive rats. *Proc Natl Acad Sci USA* 1998, 95:4754–4759
16. Reiter CD, Teng RJ, Beckman JS: Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. *J Biol Chem* 2000, 275:32460–32466
17. Zou MH, Leist M, Ullrich V: Selective nitration of prostacyclin synthase and defective vasorelaxation in atherosclerotic bovine coronary arteries. *Am J Pathol* 1999, 154:1359–1365
18. Hung TH, Skepper JN, Burton GJ: In vitro ischemia-reperfusion injury in term human placenta as a model for oxidative stress in pathological pregnancies. *Am J Pathol* 2001, 159:1031–1043
19. Baker CS, Hall RJ, Evans TJ, Pomerance A, Macclouf J, Creminon C, Yacoub MH, Polak JM: Cyclooxygenase-2 is widely expressed in atherosclerotic lesions affecting native and transplanted human coronary arteries and colocalizes with inducible nitric oxide synthase and nitrotyrosine particularly in macrophages. *Arterioscler Thromb Vasc Biol* 1999, 19:646–655
20. Mohazzab KM, Kaminski PM, Wolin MS: NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. *Am J Physiol* 1994, 266:H2568–H2572
21. Huang A, Sun D, Kaley G, Koller A: Superoxide released to high intra-arteriolar pressure reduces nitric oxide-mediated shear stress- and agonist-induced dilations. *Circ Res* 1998, 83:960–965
22. Daiber A, Herold S, Schoneich C, Namgaladze D, Peterson JA, Ullrich V: Nitration and inactivation of cytochrome P450BM-3 by peroxynitrite. Stopped-flow measurements prove ferryl intermediates. *Eur J Biochem* 2000, 267:6729–6739
23. Mohazzab KM, Wolin MS: Sites of superoxide anion production detected by lucigenin in calf pulmonary artery smooth muscle. *Am J Physiol* 1994, 267:L815–L822
24. Elliott SJ, Lacey DJ, Chilian WM, Brzezinska AK: Peroxynitrite is a contractile agonist of cerebral artery smooth muscle cells. *Am J Physiol* 1998, 275:H1585–H1591
25. Garcia Soriano F, Virag L, Jagtap P, Szabo E, Mabley JG, Liaudet L, Marton A, Hoyt DG, Murthy KG, Salzman AL, Southan GJ, Szabo C: Diabetic endothelial dysfunction: the role of poly(ADP-ribose) polymerase activation. *Nat Med* 2001, 7:108–113
26. Bellamy MF, McDowell IF, Ramsey MW, Brownlee M, Bones C, Newcombe RG, Lewis MJ: Hyperhomocysteinemia after an oral methionine load acutely impairs endothelial function in healthy adults. *Circulation* 1998, 98:1848–1852
27. Tawakol A, Omland T, Gerhard M, Wu JT, Creager MA: Hyperhomocyst(e)inemia is associated with impaired endothelium-dependent vasodilation in humans. *Circulation* 1997, 95:1119–1121
28. Chao CL, Kuo TL, Lee YT: Effects of methionine-induced hyperhomocysteinemia on endothelium-dependent vasodilation and oxidative status in healthy adults. *Circulation* 2000, 101:485–490
29. Kanani PM, Sinkey CA, Browning RL, Allaman M, Knapp HR, Haynes WG: Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans. *Circulation* 1999, 100:1161–1168
30. Nappo F, De Rosa N, Marfella R, De Lucia D, Ingrosso D, Perna AF, Farzati B, Giugliano D: Impairment of endothelial functions by acute hyperhomocysteinemia and reversal by antioxidant vitamins. *JAMA* 1999, 281:2113–2118
31. Welch GN, Upchurch Jr GR, Farivar RS, Pigazzi A, Vu K, Brecher P, Keaney Jr JF, Loscalzo J: Homocysteine-induced nitric oxide production in vascular smooth-muscle cells by NF-kappa B-dependent transcriptional activation of Nos2. *Proc Assoc Am Physicians* 1998, 110:22–31
32. Blundell G, Jones BG, Rose FA, Tudball N: Homocysteine mediated endothelial cell toxicity and its amelioration. *Atherosclerosis* 1996, 122:163–172
33. Weiss N, Zhang YY, Heydrick S, Bierl C, Loscalzo J: Overexpression of cellular glutathione peroxidase rescues homocyst(e)ine-induced endothelial dysfunction. *Proc Natl Acad Sci USA* 2001, 98:12503–12508
34. Wolin MS: Interactions of oxidants with vascular signaling systems. *Arterioscler Thromb Vasc Biol* 2000, 20:1430–1442
35. Zhang X, Li H, Jin H, Ebin Z, Brodsky S, Goligorsky MS: Effects of homocysteine on endothelial nitric oxide production. *Am J Physiol* 2000, 279:F671–F678
36. Lassegue B, Sorescu D, Szocs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK: Novel gp91(phox) homologues in vascular smooth muscle cells: nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ Res* 2001, 88:888–894
37. Sorescu D, Somers MJ, Lassegue B, Grant S, Harrison DG, Griendling KK: Electron spin resonance characterization of the NAD(P)H oxidase in vascular smooth muscle cells. *Free Radic Biol Med* 2001, 30:603–612
38. Gupte SA, Okada T, Tateyama M, Ochi R: Activation of TxA2/PGH2 receptors and protein kinase C contribute to coronary dysfunction in superoxide treated rat hearts. *J Mol Cell Cardiol* 2000, 32:937–946
39. van der Loo B, Labugger R, Skepper JN, Bachschmid M, Kilo J, Powell JM, Palacios-Callender M, Erusalimsky JD, Quaschnig T, Malinski T, Gygi D, Ullrich V, Lüscher TF: Enhanced peroxynitrite formation is associated with vascular aging. *J Exp Med* 2000, 192:1731–1744
40. Rossig L, Fichtlscherer B, Breitschopf K, Haendeler J, Zeiher AM, Mulsch A, Dimmeler S: Nitric oxide inhibits caspase-3 by S-nitrosation in vivo. *J Biol Chem* 1999, 274:6823–6826
41. Poddar R, Sivasubramanian N, DiBello PM, Robinson K, Jacobsen DW: Homocysteine induces expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human aortic endothelial cells: implications for vascular disease. *Circulation* 2001, 103:2717–2723
42. Voutilainen S, Morrow JD, Roberts LJ, Alfthan G, Alho H, Nyyssonen K, Salonen JT: Enhanced in vivo lipid peroxidation at elevated plasma total homocysteine levels. *Arterioscler Thromb Vasc Biol* 1999, 19:1263–1266
43. Southern F, Eidt J, Drouilhet J, Mukunyadzi P, Williams DK, Cruz C, Wang YF, Poirier LA, Brown AT, Moursi MM: Increasing levels of dietary homocystine with carotid endarterectomy produced proportionate increases in plasma homocysteine and intimal hyperplasia. *Atherosclerosis* 2001, 158:129–138