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Chronic high blood flow potentiates shear stress-induced release of NO in arteries of aged rats

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Abstract

Aging impairs shear-stress-dependent dilation of arteries via increased superoxide production, decreased SOD activity, and decreased activation of endothelial nitric oxide (NO) synthase (eNOS). In the present study, we investigated whether chronic increases in shear stress, elicited by increases in blood flow, would improve vascular endothelial function of aged rats. To this end, second-order mesenteric arteries of young (6 mo) and aged (24 mo) male Fischer-344 rats were selectively ligated for 3 wk to elevate blood flow in a first-order artery [high blood flow (HF)]. An *in vitro* study was then conducted on first-order arteries with HF and normal blood flow (NF) to assess shear stress (1, 10, and 20 dyn/cm²)-induced release of NO into the perfusate. In HF arteries of both age groups, shear stress-induced NO production increased significantly. In 24-mo-old rats, the reduced shear stress-induced NO production in NF arteries was normalized by HF to a level similar to that in NF arteries of 6-mo-old rats. The increased NO production in HF arteries of 24-mo-old rats was associated with increased shear stress-induced dilation, expression of eNOS protein, and shear stress-induced eNOS phosphorylation. Wortmannin, a phosphatidylinositol 3-kinase inhibitor, reduced shear stress-induced eNOS phosphorylation and vasodilation. Superoxide production decreased significantly in HF compared with NF arteries in 24-mo-old rats. The decreased superoxide production was associated with significant increases in CuZn-SOD and extracellular SOD protein expressions and total SOD activity. These results suggest that stimulation with chronic HF restores shear-stress-induced activation of eNOS and antioxidant ability in aged arteries.

Keywords

endothelium; endothelial nitric oxide synthase phosphorylation; arterial ligation

Endothelium-dependent vasodilator responses in small arteries and arterioles play a significant role in the regulation of local tissue perfusion and systemic blood pressure. *In vivo*, the endothelium-dependent dilation is mainly elicited by blood flow-generated shear stress, which stimulates the endothelium to release vasodilators, such as nitric oxide (NO), to regulate vessel diameter. Decreased flow/shear-stress-induced dilation is now recognized

as the hallmark of endothelial dysfunction in animals and humans in many pathological conditions. On the other hand, an increased flow-induced dilation accompanies chronic exercise activity (25). The intermittent increases in blood flow during exercise, which increase shear stress stimulation of the endothelium, is believed to be one of the fundamental mechanisms responsible for the exercise-induced improvement of endothelial function (24). Studies have also demonstrated that increases in blood flow by an arteriovenous fistula enhance endothelium-dependent dilation and the release of endothelium-derived dilator factors (13, 14). Furthermore, recent studies have shown that chronic increases in blood flow, in the aorta caused by an aortocaval fistula (15) and in the mesenteric artery by selective arterial ligation (28) in rats, enhance endothelial NO synthase (eNOS) mRNA and protein expression, whereas a reduction in blood flow decreases eNOS expression (4).

Aging is accompanied by endothelial dysfunction. In our previous study (26), we found that shear stress-induced dilation and release of NO in mesenteric arterioles and arteries of 24-mo-old rats were significantly reduced due to decreased shear stress-induced eNOS phosphorylation, increased superoxide ($O_2^{\cdot-}$) production, and decreased SOD activity. The purpose of the present study, therefore, was to examine whether an increase in blood flow, by selective mesenteric artery ligation, can restore endothelial function in aged vessels. Thus, we compared shear stress-induced release of NO, eNOS expression and phosphorylation, SOD expression and activity, $O_2^{\cdot-}$ production in isolated mesenteric arteries of young and aged rats.

Materials and Methods

Animals

Male Fischer-344 rats (age: 6 and 24 mo) were used. All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the current guidelines of the National Institutes of Health and the American Physiological Society for the care and use of laboratory animals.

Arterial ligations and blood flow measurements

Rats were anesthetized by injections of pentobarbital sodium (50 mg/kg) intramuscularly and placed on a heating pad to maintain body temperature at 37°C. Surgery for the ligation of mesenteric arteries was similar to that reported by Pourageaud and De Mey (17). Briefly, an 8th to 10th first-order artery proximal to the ileocecal junction was located. A first-order artery was finally selected based on its size and length using a microruler in the eyepiece of a dissecting microscope to match vessels of 6- and 24-mo-old rats. High blood flow (HF) in the artery was elicited by ligation of two second-order branches of the adjacent first-order artery, one on each side of the artery (Fig. 1). A 3rd to 5th first-order artery proximal to the HF artery was selected as the normal blood flow (NF) control. Three weeks after surgery, HF and NF arteries were isolated for in vitro experiments.

In a group of rats, blood flow in HF arteries was measured by a 1-mm ultrasonic flow probe and a TS420 flowmeter (Transonic System), before, immediately after, and 3 wk after the

ligation. Changes in blood flow were recorded, and the average flow rate was calculated from a minimum recording period of 5 min.

Shear stress and perfusate nitrite

Similar to our previous study (26), NF and HF first-order arteries were isolated from young (6 mo old) and aged (24 mo old) rats. Arteries were cannulated in a vessel chamber and perfused and suffused with MOPS-buffered physiological salt solution (PSS) at 37°C and pH 7.4. Intravascular pressure was maintained constant at 80 mmHg. The internal diameter was measured with a video caliper. The average diameter of the vessels was calculated by measuring the diameters along the entire length of the vessels at 500- μ m intervals. The length of the isolated arteries was also measured. Wall shear stresses of 1, 10, and 20 dyn/cm² were established by increasing perfusate flow using a syringe pump (Harvard Apparatus) and calculated based on the average diameter of each vessel. Perfusate (200 μ l) at the outflow tubing was collected at each level of shear stress under control conditions and after the administration of *N*⁰-nitro-L-arginine methyl ester (L-NAME 3×10^{-4} M), an inhibitor of NOS. Vessels were reequilibrated for 10 min at zero intraluminal flow before subsequent levels of shear stress were applied. Nitrite formation in the perfusate was assessed by a fluorometric assay with a spectrofluorometer (12). Standard curves of nitrite (0–640 μ M) were constructed using MPOS as a vehicle. Nitrite production in response to shear stress was expressed as picomoles per millimeter squared of the luminal surface of the artery per minute.

Shear stress-induced dilation

The distal ends of first-order mesenteric arteries, ~ 3 –4 mm in length, were isolated and perfused in the vessel chamber as described above. Arteries were precontracted with phenylephrine (10^{-8} – 5×10^{-8} M) to 40–50% of their maximal diameter. An initial shear stress of 10 or 20 dyn/cm² was then applied to the vessels. Shear-stress-induced dilation was assessed under control conditions and in the presence of L-NAME (3×10^{-4} M). The initial shear stress was obtained by applying an intraluminal flow calculated according to the diameter obtained before the application of shear stress as follows: $\tau = 4Q\eta/\pi r^3$, where τ is the initial shear stress, Q is intraluminal flow, r is the internal radius of the vessel, and η is the viscosity of PSS (equal to 0.0069 Poise at 37°C).

In separate experiments, shear-stress (20 dyn/cm²)-induced dilation in HF arteries of 6- and 24-mo-old rats was assessed before and after the administration of wortmannin (10^{-7} M).

O₂^{•-} production

O₂^{•-} production was determined using the nitroblue tetrazolium (NBT) reduction assay. Vessels were cannulated and equilibrated at 80 mmHg for 60 min, followed by intra- and extraluminal administration of NBT (10^{-5} M) for 30 min. Vessels were then rinsed intra- and extraluminally with cold (4°C) PSS for 5 min to stop the reaction and remove extra NBT. Vessels were then removed from the cannula and placed immediately into 2 M KOH overnight to allow the vessels to be digested completely. The samples were further mixed with DMSO at a ratio of 1:1.2. Negative controls were established by adding SOD (100 U/ml) with NBT to the vessels for the same period of time. O₂^{•-} formation in the vessels

was determined spectrophotometrically at 630 nm (18). The SOD-inhibitable NBT reduction was calculated by subtracting the average of the negative controls from all other samples. The final $O_2^{\cdot -}$ production was expressed as nmoles of NBT per millimeter squared of the internal surface area of the vessel per 30 min of incubation time.

Western blot analysis

After perfusion, arteries were pulverized in liquid nitrogen. Samples were solubilized in lysis buffer containing 1% protease inhibitor cocktail (Sigma) in ice for 30 min followed by two 1-min sonication with a 5-min interval. Samples containing 10 μ g protein were separated on 10% SDS-PAGE gels, transferred to a polyvinylidene difluoride membrane, and probed with primary antibodies of Cu/Zn-SOD, Mn-SOD, or extracellular (EC-)SOD and secondary antibodies according to the Amersham ECL-Plus protocol. β -Actin was used to normalize for loading variations.

In separate experiments, a shear stress of 20 dyn/cm² was applied to the arteries for 20 min. Single arteries were then removed from the cannulae, pulverized in liquid nitrogen, and homogenized in Laemmli buffer directly with 1% of protease and phosphatase inhibitor cocktails (Sigma). All proteins isolated from a single vessel were used as a sample for Western blot analysis of phospho-eNOS (p-eNOS) and eNOS. Specific bands of p-eNOS and eNOS were normalized by the internal surface area of each vessel. Shear stress (20 dyn/cm²)-induced p-eNOS of HF arteries was also assessed in the absence and presence of wortmannin.

SOD activity

Total SOD activity of the arteries was assessed by measuring the inhibition of pyrogallol autoxidation (26); 20 μ g protein of arteries was used, and the reaction was monitored spectrophotometrically (420 nm) at room temperature for 3 min. The activity was calculated against a standard curve of SOD (0–1.6 U/ml, S-2515, Sigma).

Statistical analysis

Data are expressed as means $\pm$ SE. *n* refers to the number of rats from which mesenteric arteries were isolated. Statistical significance was calculated by repeated-measures ANOVA followed by a Tukey/Kramer multiple-comparison test. Student's *t*-test was also used as appropriate. The significance level was taken at *P* < 0.05.

Results

Selective ligation of second-order mesenteric arteries significantly increased blood flow in first-order arteries of 6- and 24-mo-old rats (Table 1). The elevated blood flow remained at a high level for 3 wk. Internal diameters of HF arteries were significantly larger than those of NF arteries in both 6- and 24-mo-old rats (Table 2).

Nitrite production in the perfusate in response to 1, 10, and 20 dyn/cm² of shear stress was measured in NF and HF arteries of 6- and 24-mo-old rats (Fig. 2). Similar to our previous findings (26), nitrite production in NF arteries was greatly reduced in 24-mo-old rats

compared with 6-mo-old rats. In HF arteries, 3 wk of HF stimulation, however, significantly increased nitrite production in both groups. At 20 dyn/cm² shear stress, nitrite production was increased by 2.7- and 2-fold in vessels of 6- and 24-mo-old rats, respectively. More importantly, HF increased nitrite production in arteries of 24-mo-old rats to a level similar to that in NF arteries of 6-mo-old rats (23 and 18 pmol·mm⁻²·min, respectively). The enhanced shear stress-induced nitrite production in HF arteries of both groups was eliminated by L-NAME (Fig. 3), suggesting that the response is eNOS dependent.

To confirm that HF improves endothelial function in arteries of aged rats, shear stress-induced dilation was assessed in NF and HF arteries of 6- and 24-mo-old rats. Figure 3 shows that initial shear stress (10 and 20 dyn/cm²)-induced dilation was significantly less in NF arteries of 24-mo-old rats compared with 6-mo-old rats. L-NAME did not inhibit shear stress-induced dilation in NF arteries of 24-mo-old rats, suggesting an impaired NO-mediated dilation in aged vessels. HF stimulation, however, significantly increased shear stress-induced dilation in both age groups. Although the dilation in HF arteries of 24-mo-old rats was significantly less compared with 6-mo-old rats, the dilation was similar to that in NF arteries of 6-mo-old rats. L-NAME significantly reduced the response in HF arteries of 24-mo-old rats. Together, these data suggest that HF improves endothelial function in aged vessels via a NO-dependent mechanism.

To analyze the underlying mechanism responsible for the enhanced nitrite production and increased shear stress-induced dilation in HF arteries, eNOS and shear stress-induced p-eNOS protein were assessed (Fig. 4). Figure 4A shows a representative Western blot detecting p-eNOS and eNOS in arteries exposed to 20 dyn/cm² shear stress for 20 min. Figure 4B shows that eNOS protein was not different in NF arteries of 6- and 24-mo-old rats but that p-eNOS was reduced in aged rats, resulting in a decreased p-eNOS-to-eNOS ratio. In HF-stimulated arteries, eNOS protein was increased in both groups of rats. Shear stress-induced p-eNOS increased significantly in HF arteries of 24-mo-old rats. The level of p-eNOS was not as high as that in HF arteries of 6-mo-old rats but was significantly higher than that in NF arteries of 6-mo-old rats. Figure 5 demonstrates that shear stress-induced phosphorylation of eNOS (A) and arterial dilation (B) in HF arteries were inhibited by wortmannin, indicating further that the dilator response is activated by the shear stress-phosphatidylinositol 3-kinase (PI3K)/Akt-p-eNOS pathway.

O₂^{·-} production was measured in pressurized arteries using the NBT reduction assay. Figure 6A shows that at 80 mmHg of intravascular pressure, O₂^{·-} production in aged NF arteries was significantly increased compared with that in arteries of young rats. This was associated with decreased EC-SOD protein expression (Fig. 6, B and C) and decreased total SOD activity (Fig. 6D). HF doubled CuZn-SOD and EC-SOD protein expression and increased SOD activity by 50% in arteries of 6-mo-old rats. In aged HF arteries, O₂^{·-} production decreased significantly compared with NF arteries but was still significantly higher than that of young rats (Fig. 6A). CuZn-SOD and EC-SOD were increased in vessels of aged HF rats, although the increase was relatively less than in those of young rats (Fig. 6C); however, SOD activity was greatly increased and became comparable with that in NF arteries of young rats (Fig. 6D).

Discussion

Several studies have demonstrated that selective arterial ligation is a reliable method to generate HF in first-order mesenteric arteries of rats. Pourageaud and De Mey showed that blood flow remained at an elevated level for 4 wk after arterial ligation (17). Similarly, in the present study, blood flow doubled immediately after the ligation and remained at an elevated level for 3 wk in both 6- and 24-mo-old rats (Table 1). Since HF induces vascular remodeling, the increased diameter in HF arteries may diminish the increased shear stress. Based on data shown in Tables 1 and 2 and assuming that the diameter of HF arteries before the ligation was not different from that of NF arteries, the calculated shear stress in HF arteries after 3-wk ligation was still 50% higher than that in HF arteries before ligation. Therefore, even in the presence of vascular remodeling, it is reasonable to assume that the elevated blood flow provided a continuously elevated shear stress in the arteries of both young and aged rats for a 3-wk duration. In addition, a single first-order mesenteric artery provides sufficient endothelial area to assess shear stress-induced release of NO. Considering that vascular remodeling may change the ratio of tissue components, the luminal surface area of vessels, instead of protein content, was used to normalize shear stress-induced NO release and eNOS phosphorylation.

As expected, release of nitrite and endothelium-dependent dilation, as a function of shear stress, were significantly attenuated in vessels of aged compared with young rats (Figs. 2 and 3). These results are consistent with our previous findings (26). Age-related changes in eNOS expression are still controversial. In coronary arterioles, eNOS mRNA and protein are reduced (1), whereas in skeletal muscle arterioles, eNOS protein is reported to be increased (23). In mesenteric arteries, however, we found in the present study as well as in our previous study (26) that eNOS protein expression and basal activity were similar in young and aged rats, whereas eNOS phosphorylation in response to shear stress was significantly reduced in vessels of aged rats. These results indicate that the reduced shear stress-induced release of NO in aged mesenteric arteries is mainly due to a reduced activation of eNOS. It is known that shear stress-induced release of NO is exclusively attributed to eNOS phosphorylation by PI3K/Akt (2, 6, 7). In cultured endothelial cells, laminar fluid shear stress time dependently initiates Akt phosphorylation via a PI3K-mediated pathway (2), followed by a significant increase in Ser¹¹⁷⁷ phosphorylation of eNOS (6). Similarly, in the present study, inhibition of PI3K with wortmannin reduced eNOS phosphorylation and vasodilation in HF arteries. It is also known that vascular stiffness progresses during the process of aging (29). A causal relationship between vascular stiffness and reduced Akt and eNOS phosphorylation has been suggested by a recent study (22), which assessed shear-stress-induced NO release in the aorta of aged rats. Similar results were also found, showing that Akt/eNOS phosphorylation was reduced in the lining of endothelial cells in stiff tubes compared with those lining compliant tubes (16). Thus, an impaired mechanotransduction to shear stress via reduced Akt/eNOS phosphorylation may play an important role in age-dependent endothelial dysfunction. Conversely, we found that high shear stress increases eNOS expression and phosphorylation, resulting in enhanced shear stress-induced release of NO and vasodilation.

Multiple signal transduction pathways are responsible for the shear stress-initiated upregulation of eNOS (7, 20). It is known that chronic HF increases eNOS gene and protein expression (15, 28) and increases endothelium-dependent dilation (11, 14). Furthermore, exercise activity, which also upregulates eNOS in response to increased blood flow (21, 30), restores endothelial function in skeletal muscle arterioles of aged rats (23). The exact mechanisms responsible for the shear stress-induced upregulation of eNOS in aged vessels have not yet been clarified. A recent study (32) demonstrated that eNOS mRNA expression was enhanced in soleus muscle feed arteries of aged rats exposed to 4 h of increased shear stress, whereas our data demonstrate that eNOS protein and eNOS phosphorylation were increased in aged vessels by chronic HF. Together, these results suggest that age-related endothelial dysfunction could be reversed by elevated shear stress.

A decreased bioavailability of NO due to the presence of greater oxidative stress contributes to endothelial dysfunction in aging (5, 27). Aging increases NADPH oxidase-dependent basal $O_2^{\cdot-}$ production in coronary vessels (1). We have previously shown that shear stress-induced NO production could be augmented by tiron, which scavenges $O_2^{\cdot-}$, suggesting there is shear stress-activated $O_2^{\cdot-}$ production, particularly in aged vessels (26). We have also demonstrated that multiple $O_2^{\cdot-}$ -producing systems (namely, NADPH oxidase, xanthine oxidase, and NOS) are responsible for pressure-induced $O_2^{\cdot-}$ production in mesenteric arteries of aged rats (9). In the present study, we further demonstrated that the age-related increase in $O_2^{\cdot-}$ production and decreases in SOD expression and activity can be reversed by chronic HF. It is plausible then that the increased levels of SOD proteins may play a significant role in the reduced $O_2^{\cdot-}$ production in aged vessels. It has been reported that shear stress increases CuZn-SOD mRNA expression and activity in cultured endothelial cells and isolated arterioles (3, 8, 31). Similar results have also been observed in exercise-induced adaptation of endothelial function (10, 19). However, the specific signaling cascade mediating the shear stress-induced upregulation of SOD in aged vessels needs to be further characterized.

In conclusion, shear-stress-induced release of NO and dilation were significantly attenuated in mesenteric arteries of aged rats. Chronic increases in blood flow markedly enhanced the release of NO in response to shear stress in aged vessels, as a result of increased eNOS and eNOS phosphorylation. Chronic increases in blood flow also significantly decreased $O_2^{\cdot-}$ formation and increased antioxidant activity in aged vessels. Together, by these mechanisms, endothelium-dependent vasodilator responses were greatly increased in chronic HF-stimulated aged vessels. Thus, our results lend credibility to the hypothesis that stimulation by shear stress improves endothelial function via increasing NO bioavailability. Many cardiovascular diseases, such as hypertension, diabetes, and atherosclerosis, share various features with aging associated endothelial dysfunction. Thus, our results are suggestive of a role of increases in blood flow to act as an efficient stimulus to improve endothelial function in aging as well as other cardiovascular diseases.

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Fig. 1.
Ligation-induced high blood flow (HF) in first-order mesenteric arteries of rats.

Fig. 2.

Shear-stress-induced perfusate nitrite in normal blood flow (NF) and HF first-order mesenteric arteries of 6-mo-old (6M; *A*) and 24-mo-old (24M; *B*) rats and in HF arteries of 6M (*C*) and 24 M (*D*) rats before and after inhibition of nitric oxide (NO) synthesis with *N*^ω-nitro-L-arginine methyl ester (L-NAME; 3×10^{-4} M). Data are normalized by the luminal surface area of each vessel. * $P < 0.05$ vs. corresponding NF data; # $P < 0.05$ vs. corresponding NF data; ‡ $P < 0.05$ vs. control.

Fig. 3.

Shear stress-induced dilation in NF and HF first-order mesenteric arteries of 6M and 24M male Fischer-344 rats before (control) and after inhibition of NO synthesis with L-NAME. An initial shear stress of 10 or 20 dyn/cm² was applied to vessels by constant intraluminal flow calculated according to their initial diameter. Values are means $\pm$ SE; $n = 6$ in each group. %PD, percent passive diameter. * $P < 0.05$ vs. corresponding NF data; # $P < 0.05$ vs. corresponding data of 6M rats; ‡ $P < 0.05$ vs. control.

Fig. 4.

A: representative Western blots of endothelial NO synthase (eNOS) and shear stress (20 dyn/cm²)-induced phospho-eNOS (p-eNOS) in NF and HF arteries of 6M and 24M rats. **B:** summary data of three Western blots (6–7 rats/group). Densitometry was normalized by the luminal surface area (A) of each vessel. Data are normalized by means of densitometry from NF arteries of 6M rats. **P* < 0.05 vs. corresponding NF data; #*P* < 0.05 vs. corresponding data of 6M rats; ‡*P* < 0.05 vs. NF data of 6M rats.

Fig. 5. Effects of wortmannin (WTM; 10^{-7} M) on shear-stress (20 dyn/cm²)-induced eNOS phosphorylation (A) and vasodilation (B) in HF arteries of 6M and 24M rats. * $P < 0.05$ vs. control.

Fig. 6.

Superoxide production (A), SOD protein expression (B and C), and total SOD activity (D) in NF and HF arteries of 6M and 24M rats. Superoxide was assessed at 80 mmHg of intravascular pressure by the nitroblue tetrazolium (NBT) reduction assay. CuZn-SOD, Mn-SOD, and extracellular (EC)-SOD were assessed by Western blot analysis. β-Actin was used to normalize for loading variations. Three blots were assessed (6 rats/group). Data are normalized by means of densitometric ratios from NF arteries of 6M rats. SOD activity was determined in single arteries by inhibition of pyrogallol autoxidation. Units of SOD were calculated using a SOD standard curve. Numbers in parentheses indicate numbers of rats used per group. * $P < 0.05$ vs. corresponding NF data; # $P < 0.05$ vs. corresponding data of 6M rats.

Table 1
Selective arterial ligation increases blood flow in first-order mesenteric arteries

	<i>n</i>	Control	Immediately After Ligation	3 wk After Ligation
6-mo-old rats	6	0.61 ±0.04	1.33±0.19 *	1.27 ±0.09 *
24-mo-old rats	4	0.64±0.03	1.31 ±0.08 *	1.24±0.11 *

Values are means ± SE (in ml/min); *n*, no. of rats/group.

* $P < 0.05$ vs. control.

Table 2
Average internal diameters of rat first-order mesenteric arteries

	6-mo-old Rats		24-mo-old Rats	
	NF	HF	NF	HF
<i>n</i>	7	15	9	13
Diameter, μm	447 $\pm$ 11	498 $\pm$ 14*	431 $\pm$ 18	469 $\pm$ 7*

Values are means $\pm$ SE; *n*, no. of rats/group. The internal diameter was measured at 80 mmHg of intravascular pressure. The average diameter was calculated by measuring the diameters along the entire length of the vessel at 500- μm intervals. NF, normal blood flow; HF, high blood flow.

* $P < 0.05$ vs. corresponding NF data.