Hypercholesterolemia impairs reactive hyperemic vasodilation of 2A but not 3A arterioles in mouse cremaster muscle

Jurgen W. G. E. VanTeeffelen, Alina A. Constantinescu, Hans Vink, and Jos A. E. Spaan
Department of Medical Physics, Cardiovascular Research Institute Amsterdam, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Submitted 23 December 2004; accepted in final form 16 February 2005


Hypercholesterolemia and atherosclerosis have been associated with changes in the microvasculature, in particular with endothelial dysfunction. In the present study, the impact of atherogenic conditions on arteriolar vasomotor control was determined. Arteriolar [second-order (2A) and third-order (3A) arterioles; diameter range: 9–37 μm] responses during reactive hyperemia (RH) were determined in cremaster muscle of anesthetized mice. C57Bl/6 mice on normal rodent chow were used as controls and high-fat/high-cholesterol (HFC)-fed C57Bl/6 and ApoE3-Leiden mice as hypercholesterolemic mice. The HFC diet resulted in time-dependent increases in plasma cholesterol and triglyceride concentrations (P < 0.001), which were more pronounced in ApoE3-Leiden mice (P < 0.001). In control mice, inhibition of nitric oxide (NO) synthesis with Nω-nitro-l-arginine (l-NNA) reduced baseline diameter from 17.9 ± 1.2 to 15.9 ± 1.3 μm (P < 0.05) and decreased the duration of RH [time to 50% (t50) of recovery: 23.3 ± 3.6 vs. 12.5 ± 1.3 s (P = 0.003)]. t50 was longer in 2A versus 3A arterioles (33 ± 3 vs. 18 ± 2 s, P < 0.001) and increased with wall shear rate at the beginning of RH in 2A arterioles only. Compared with control mice, RH responses were reduced in 2A arterioles of HFC mice (t50: 11 ± 2 s, P < 0.001 vs. control) but not affected in 3A vessels. l-NNA did not affect baseline diameter in HFC mice and reduced t50 only in “slow” responders (t50 ≈ 10 s). It is concluded that hypercholesterolemia results in an impairment of NO-mediated vasomotor control in 2A but not 3A arterioles during dynamic changes of perfusion like RH. 2A arterioles likely therefore represent the functional locus of endothelial dysfunction during atherogenic conditions.

endothelial dysfunction; shear-dependent dilation; arteriolar heterogeneity; ApoE3-Leiden

Hypercholesterolemia is a primary risk factor for coronary artery disease. Although atherosclerosis itself is confined to larger sized arteries, vascular changes extend into the microcirculation (18, 20, 33). In patients and animal models, hypercholesterolemia and atherosclerosis have been generally associated with an endothelial dysfunction of the resistance vasculature, representing a diminished bioavailability of nitric oxide (NO) as reflected by an impaired agonist-induced endothelium-dependent flow increase (3, 8). This endothelial dysfunction is found in early stages of atherosclerosis and precedes overt atherosclerosis (8, 26, 37). The impairment of microvascular function in hypercholesterolemia has been indicated to compromise coronary blood flow regulation and promote ischemia in the absence of a flow-limiting stenosis (28, 36).

Studies in microcirculatory preparations of hypercholesterolemic animals have confirmed the occurrence of endothelial dysfunction by showing impairment of arteriolar vasodilation to NO-dependent agonists (26, 35). However, at the moment there is no information on how such impairment would interfere with normal physiological function. In any case, understanding the progression of microvascular disease during hypercholesterolemia necessitates functional changes at the level of the microvascular network to be identified. Because resistance vessels show heterogeneity in their response to hemodynamic and metabolic stimuli (13, 19, 22, 25), it might well be that the effect of hypercholesterolemia on microvascular function depends on location in the resistance network, an aspect that typically has not been considered in previous studies.

In the present study, we studied the effect of hypercholesterolemia on the vasomotor response of two distinct branches of the arteriolar network during reactive hyperemia (RH). RH responses of second-order (2A) and third-order (3A) vessels were measured in cremaster muscle (15, 22, 23) of control C57Bl/6 mice and hypercholesterolemic mice. To induce hypercholesterolemia, we placed C57Bl/6 mice and transgenic ApoE3-Leiden mice on a high-fat/high-cholesterol (HFC) diet. The transgenic mice express ApoE3-Leiden, a dysfunctional human apolipoprotein E (ApoE) variant that causes dysbetalipoproteinemia in mutants (29). A HFC diet strongly exacerbates the hyperlipidemia and results in overt atherosclerosis in these mice (10, 30, 31). Furthermore, in ApoE3-Leiden mice on a HFC diet, endothelial dysfunction of cremaster arterioles was evident from the impaired relaxation to bradykinin (4). We hypothesized in the present study that atherogenic conditions would result in impaired NO-mediated vasodilation during RH. Because shear stress is an important physiological stimulus for NO release in the microcirculation (16, 27), we hypothesized that the impairment would be prominent in the proximal, shear stress-sensitive, resistance vessels compared with the distal, more metabolically influenced, arterioles.

METHODS

*Mice and Lipid Status*

All procedures and protocols were approved by the Animal Care and Use committee of the Academic Medical Center. Experiments were performed on male mice (25–30 g body wt). C57Bl/6 mice were obtained from Charles River Europe. ApoE3-Leiden mice, transgenic strain 2, were obtained from the Gaubius Laboratorium TNO-PG...
(Leiden, The Netherlands) and were cross-bred with C57Bl/6 mice (29, 30); transgenic animals of the F10-F11 generation, identified by PCR analysis of genomic DNA from the ear pavilion, were used for the current experiments. Control C57Bl/6 mice received standard rat/mouse chow (AM-2, Hope Farms; Woerden, The Netherlands). To induce hyperlipidemia, C57Bl/6 mice and ApoE3-Leiden mice were placed at the age of ~8 wk on a cholesterol-enriched high-fat diet (0.5% cholate, 15% cocoa butter, 1% cholesterol, 40.5% sucrose, 10% corn starch, 1% corn oil, and 4.7% cellulose; HFC 0.5% diet, Hope Farms). To determine the effect of the diet on circulating lipid levels, plasma cholesterol and triglyceride concentrations were determined in C57Bl/6 (n = 24) and ApoE3-Leiden (n = 22) mice when fed standard chow and at different time points after placement on the 0.5% HFC diet. Plasma lipids were measured enzymatically using commercial kits (cholesterol: no. 236691, Boehringer-Mannheim; triglycerides: no. 337-B, Sigma Diagnostics). Lipid analyses were performed in other mice than the ones used for the cremaster studies (see Cremaster Preparation and Video Microscopy).

Cremaster Preparation and Video Microscopy

Male mice (n = 48 total) were anesthetized with an intraperitoneal injection of ketamine hydrochloride (125 mg/kg) and medetomidine (0.2 mg/kg) and tracheotomized (polyethylene-90 tubing) to ensure airway patency. The depth of anesthesia was maintained according to the stability of blood pressure, respiration rate, and lack of pedal withdrawal reflex by supplemental administration (every ~1 h) of anesthetic (ketamine: 15 mg/kg ip; medetomidine: 35 µg/kg ip). To countereffect decreases in blood pressure and heart rate induced by medetomidine (38), atropine was administered at regular intervals (~1 h) during the experiment (initial dose: 0.5 mg/kg sc; maintenance: 0.125 mg/kg ip). The right carotid artery was cannulated (polyethylene-10 tubing) for monitoring of systemic blood pressure. Esophageal temperature was maintained at ~37°C by radiant heat. At the end of the experimental procedures, the mouse was given an overdose of ketamine.

The mouse was placed in a supine position on a custom-built animal platform. The right cremaster muscle was prepared as originally described by Baez (1). Briefly, an incision was made through the skin, and the muscle was dissected from the surrounding connective tissue. The exposed muscle was positioned on a clear Silicon pedestal and longitudinally incised from the apex to the inguinal canal with minimal disruption of the vascular supply. After the deferralarterial and vein were severed, the testis and epididymis were dissected away and repositioned in the abdominal cavity. The cremaster muscle was spread radially on the pedestal and pinned at the edges. The muscle was continuously (~5 ml/min) superfused at 34°C with a bicarbonate-buffered physiological salt solution of the following composition (in mM): 131.9 NaCl, 4.7 KCl, 2.0 CaCl2, 1.2 MgSO4, and 20 NaHCO3, equilibrated with 5% CO2-95% N2 to obtain a pH of ~7.4.

After surgery, the completed preparation was transferred to the stage of an intravital microscope (Olympus). Arterioles were observed using bright-field (100-W Hg lamp) microscopy [condensor: Olympus MSPlan 5, numerical aperture (NA): 0.13] and an Olympus MSPlan 20 (NA: 0.40) objective. Images were coupled to a charge-coupled device video camera via a telescopic tube with adjustable length: final object-to-camera magnification ranged from ~300 to ~700. Images were displayed on a Philips CM 8833-II video monitor and recorded using a SVHS video tape recorder (JVC BR-S611E) and a time coding interface unit (JVC SA-F911E) for post hoc diameter analysis.

Experimental Protocol

The preparation was equilibrated for 30 min, during which time the arteriolar network was scanned and a schematic diagram drawn to identify sites for data collection. A first-order (1A) or 2A arteriole in the proximal and central region of the muscle was chosen as the occlusion site. With the use of a microforce, careful pulling of a glass tube (outer diameter: 1.41 mm) and bending its distal end at an angle of ~70° resulted in a microoccluder with a tip of ~25 µm diameter and ~100 µm length. With the use of a micromanipulator (Narashige), the tip was positioned horizontally above the vessel and gently lowered until it completely compressed the vessel. After 30 s, the occlusion was released, and blood flow resumed. During the course of an experiment, it was regularly verified that the occluder had not moved or that the occlusion procedure had not damaged the vessel or surrounding tissue.

In each experiment, arteriolar responses during occlusion and subsequent RH were measured at one or two randomly chosen sites in the network distal (2–10 mm) from the occlusion site. Occlusions were performed every ~10–15 min, and arteriolar responses were repeatedly (2–6 times) measured in a random fashion during an experiment. Preliminary experiments revealed that this period of time between repetitive occlusions was sufficient for resting tone to recover while obtaining reproducible responses. Arteriolar responses were obtained for a period of 60–90 min in control C57Bl/6 mice (standard chow, n = 37) and C57Bl/6 mice (0.5% HFC) and ApoE3-Leiden mice (n = 6) that had been placed on the HFC diet for 4–70 days.

NO synthesis was blocked in a number of animals by adding Nω-nitro-l-arginine (l-NNA; 10-4 M, Sigma) to the physiological salt solution superfusing the muscle (12). Arteriolar responses were re-evaluated at least 30 min after the initial addition of l-NNA. At the end of the experimental procedures, sodium nitroprusside (SNP, 10-5 M) was added to the superfusate to determine the vasodilator capacity of the arterioles studied. To verify whether maximum vasodilation was obtained with SNP, adenosine (10-5 M, Sigma) was additionally administered in a number of experiments.

Measurements and Data Analysis

Internal vessel diameter (D), measured from the internal edges of the vessel lumen, was determined off-line during video playback from the monitor using video calipers calibrated against a stage micrometer (resolution: ±2 µm). Centerline red blood cell velocity (Vrbc) was monitored on-line with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, TX). Data were acquired at 100 Hz using a PowerLab 4/SP system (AD Instruments) coupled to a Pentium-based personal computer.

For each individual occlusion, D and Vrbc were averaged for ~30 s before the occlusion (baseline), for the last 5 s of the occlusion period (end occlusion), and for every 5 s after the release of the occlusion (RH; see Fig. 2A, left). Diameters were normalized (Dnorm) from their baseline (Dnorm) to their maximum value (Dmax) during RH, i.e., Dnorm = (D - D0)/(Dmax - D0). As an index of the recovery rate of diameter, the time to 50% recovery of Dnorm after the release of the occlusion (t50) was used (Fig. 2A, right). Mean Vrbc (Vrbc) was calculated from Vrbc using a correction factor of 1.3 (5). Blood flow was calculated as π(D2/4)Vrbc. Similar to the diameter recovery, a t50 of recovery for blood flow was calculated. Wall shear rate (WSR) was calculated as Vrbc/D. WSR at the beginning of RH (Fig. 3, bottom) was determined from the mean Vrbc during the first 0.5 s after flow restoration and D at end occlusion.

Repeated observations during a given condition in an individual vessel were averaged. Arterioles were identified topologically as 1A (feed arteriole), 2A (arcade arterioles), or 3A (transverse arterioles). Time-dependent effects of the 0.5% HFC diet on plasma cholesterol and triglyceride concentrations in C57Bl/6 and ApoE3-Leiden mice were analyzed using two-way ANOVA. Two-way ANOVA was also used to evaluate the effects of branch order (1A, 2A, and 3A) and animal treatment (C57Bl/6 on chow, C57Bl/6 on HFC diet, and ApoE3-Leiden on HFC diet) on the dilator response to SNP (Fig. 1). Within each animal group, the effects of l-NNA on baseline, end occlusion and peak RH diameter, and t50 of recovery were determined using paired comparisons (Fig. 2). The effects of branch order (2A vs. 3A) and diet (control vs. 0.5% HFC) on relative dilations to end
occlusion and peak RH and t50 of recovery of diameter and blood flow were determined using two-way ANOVA (Fig. 3). Post hoc comparisons were performed using Tukey tests. Results were considered statistically significant with \( P \leq 0.05 \). Summary data are reported as means \( \pm \) SE.

**RESULTS**

**Plasma Lipid Analysis**

Total plasma cholesterol and triglyceride concentrations were determined in ApoE3-Leiden and C57Bl/6 mice after placement on the 0.5% HFC diet. Cholesterol and triglyceride levels increased with time (\( P < 0.001 \)), and their increase was larger (\( P < 0.001 \)) in ApoE3-Leiden mice compared with the C57Bl/6 mice (interaction time and animal strain: \( P < 0.001 \)). In C57Bl/6 mice, cholesterol concentrations increased from 1.5 ± 0.15 (chow, \( n = 10 \)) to 6.0 ± 0.61 mM after 3 mo of the HFC diet (\( t = 8 \)) and further to 8.3 ± 0.33 mM after 6 mo of the HFC diet (\( n = 6 \)). In these animals, triglyceride levels were not affected by the diet [0.20 ± 0.03 (chow), 0.15 ± 0.02 (3 mo), and 0.19 ± 0.01 (6 mo) mM]. In ApoE3-Leiden mice, cholesterol concentrations increased from 2.2 ± 0.24 (chow, \( n = 6 \)) to 21.6 ± 1.84 mM after 1 mo (\( n = 5 \)) and further to 30.3 ± 1.63 (\( n = 7 \)) and 33.2 ± 2.93 mM (\( n = 4 \)) after 3 and 6 mo of the HFC diet, respectively. Also, triglyceride concentrations increased in ApoE3-Leiden mice [0.19 ± 0.02 (chow), 0.20 ± 0.03 (1 mo), 1.18 ± 0.03 (3 mo), and 1.37 ± 0.13 (6 mo) mM].

**Dilation to SNP**

Figure 1 illustrates that vasodilator capacity, as inferred from the vessel relaxation during superfusion with \( 10^{-5} \) M SNP, was unaffected in HFC-fed animals compared with control mice. Subsequent addition of \( 10^{-5} \) M adenosine in the presence of SNP did not further increase arteriolar diameter (\( n = 4–12 \) vessels/group), indicating that maximum relaxation was indeed obtained during SNP. In the three animal groups, dilation to SNP increased with decreasing arteriolar size and hence was progressively greater distally, in the higher order branches (\( P < 0.001 \)), indicating that basal vasomotor tone increased from large to small vessels in this preparation.

![Figure 1](image1.png)

**Fig. 1.** Relaxation to sodium nitroprusside (SNP). Relative dilations to \( 10^{-5} \) M SNP versus baseline diameter of all arterioles studied in the present study both at occlusion and observation sites (see METHODS) are shown. SNP dilation increased with branch order (\( P < 0.001 \)) and did not differ between treatment groups. HFC diet, high-fat/high-cholesterol diet.

**Reactive Hyperemia**

Mean arterial pressure was 65–75 mmHg and heart rate was 300–400 beats/min during the experiments and did not differ between C57Bl/6 versus ApoE3-Leiden mice. Arteriolar pressure and heart rate were not changed during superfusion with L-NNA. Occlusions were performed in 1A and 2A arterioles (diameter range: 22–73 \( \mu \)m). Distal from the occlusion, arterioles started to dilate within 2–3 s after the start of occlusion and reached steady-state dilation after \( \sim 20 \) s. Upon release of the occlusion after 30 s, vessel diameter increased further, reaching a peak within 5–10 s after the occlusion, and then returned to its preocclusion value (Figs. 2 and 3). Diameters returned consistently to their resting value after an occlusion and were stable during the course of the experiment.

**Control mice.** Figure 2A, top, shows average (left; control vs. L-NNA) and normalized individual (right; control only) diameter tracings in arterioles of C57Bl/6 mice that received normal rodent chow. As demonstrated in Fig. 2A by the vertical dotted lines, the duration of vasodilation varied between arterioles, as indicated by a \( t_{50} \) of recovery that ranged from 5 (\( t_{50A} \)) to 55 s (\( t_{50B} \)). Superfusion with L-NNA (Fig. 2A, left) decreased baseline diameter from 17.9 ± 1.2 to 15.9 ± 1.3 \( \mu \)m (\( P < 0.05 \)) and the diameter at end occlusion from 24.6 ± 1.4 to 23.6 ± 1.6 \( \mu \)m (\( P = 0.05 \)). L-NNA impaired the vasodilator response during RH by decreasing the \( t_{50} \) of recovery from 23.3 ± 3.6 s to 12.5 ± 1.3 s (\( P = 0.003 \)) and by decreasing peak dilation from 29.1 ± 1.3 to 26.4 ± 1.5 \( \mu \)m (\( P < 0.01 \)). The duration of the response appeared more homogeneous among vessels after L-NNA, with the \( t_{50} \) ranging from 5 to 20 s (data not shown).

**HFC mice.** Occlusions were performed in C57Bl/6 (Fig. 2B) and ApoE3-Leiden (Fig. 2C) mice that had been placed on the HFC diet. Baseline, end occlusion, and peak RH diameters in HFC animals were comparable to those in control animals. However, postocclusion recovery seemed faster in these animals compared with mice that received standard chow. Diameters returned to baseline with a median \( t_{50} \) of recovery of 12.5 s (range: 5–30 s; Fig. 2B, right) in the HFC-fed C57Bl/6 mice and a median \( t_{50} \) of recovery of 10 s (range: 5–25 s; Fig. 2C, right) in the HFC-fed ApoE3-Leiden mice. In the latter group, an undershoot in diameter beyond baseline was observed in two vessels in the first \( \sim 15 \) s of flow restoration. In contrast to control mice, the addition of L-NNA to the superfuse did not affect arteriolar diameter at baseline in HFC mice. However, as in the control mice, peak diameter was reduced during NO blockade. In the HFC vessels that responded fast (\( t_{50} \) ≤ 10 s) before NO blockade, L-NNA superfusion resulted in the appearance of vasomotion (\( n = 2 \) of 6 vessels in each group), precluding determination of a \( t_{50} \) of recovery. In every other HFC vessel, \( t_{50} \) of recovery decreased during L-NNA.

**Branch Order Differences in Arteriolar Vasodilator Response**

Arteriolar responses in control and HFC vessels were grouped according to branch order and are presented in Fig. 3 (left: 2A arterioles; right: 3A arterioles). Because responses did not statistically differ between the C57Bl/6 mice receiving the HFC diet and HFC-fed ApoE3-Leiden mice (see Fig. 2, B and C), data of both animal groups were pooled. ANOVA revealed greater relative dilations in 3A versus 2A arterioles (end
occlusion: \( P < 0.05 \); peak RH: \( P < 0.06 \). Furthermore, \( t_{50} \) of recovery of diameter and blood flow was diminished in HFC versus control mice (diameter, \( P = 0.003 \); flow, \( P = 0.03 \)), and this effect seemed dependent on branch order (interaction between diet and branch order for diameter \( t_{50} : P = 0.015 \); for blood flow \( t_{50} : P = 0.062 \)).

2A arterioles (Fig. 3, left) of control and HFC-fed animals had similar baseline diameters, \( 22.3 \pm 1.3 \) and \( 21.3 \pm 2.0 \) \( \mu \)m, and dilated to a similar extent at end occlusion (135 \pm 6 and 138 \pm 5\% of baseline, respectively) and at peak RH (167 \pm 6 and 162 \pm 10\% of baseline; Fig. 3, top). The duration of the dilator response was, however, diminished in 2A arterioles of HFC-fed animals compared with control vessels (\( t_{50} : 11 \pm 2 \) vs. \( 33 \pm 3 \) s, \( P < 0.001 \); Fig. 3, top middle). The range of \( t_{50} \) was 5–25 s in vessels from HFC-fed animals and 5–85 s in control vessels.

3A arterioles (Fig. 3, right) of HFC-fed animals had somewhat larger baseline diameters than in control, \( 16.4 \pm 1.3 \) vs. \( 14.6 \pm 0.9 \) \( \mu \)m (\( P > 0.05 \)) but demonstrated similar dilations at end occlusion (to \( 154 \pm 8 \) and \( 154 \pm 11\% \) of baseline, respectively) and at peak RH (to \( 187 \pm 12 \) and \( 183 \pm 10\% \) of baseline; Fig. 3, top). Recovery to baseline of 3A arterioles did not differ in control and HFC with a \( t_{50} \) of 11 vs. 24 s, \( P = 0.001 \); Fig. 3, top middle). \( t_{50} \) of recovery was larger (\( P < 0.001 \)) in 2A versus 3A vessels of control mice but not in HFC-fed mice.

Blood flow responses in 2A and 3A arterioles are shown in Fig. 3, bottom middle. Similar to peak diameter, peak RH flow increases did not differ between control and HFC in 2A (299 \pm 19 vs. 321 \pm 51\% of baseline) and 3A (386 \pm 55 vs. 381 \pm 85\% of baseline) vessels. However, as with \( t_{50} \) of recovery of diameter, \( t_{50} \) of recovery of blood flow was decreased in 2A arterioles from HFC-fed animals compared with control (11 vs. 24 \pm 2 s), but similar in 3A vessels of HFC-fed and control mice (17 \pm 4 vs. 18 \pm 3 s). The dynamics of WSR during RH are presented in Fig. 3, bottom. Baseline WSRs were comparable in control and HFC vessels and were \( 1,933 \pm 172 \) and \( 2,078 \pm 294 \) s\(^{-1} \) in 2A arterioles and \( 2,758 \pm 345 \) and
moments of flow restoration to 131 sion, WSR increased in a stepwise manner in the initial WSR dropped to near-zero values. After release of the occlusion, WSR increased with initial WSR upon flow restoration in 2A arterioles (left) but not in 3A vessels (right). Data from HFC arterioles have been added to the Fig. 4 and are indicated by the open circles. Given a WSR at the beginning of RH of ~2,000 s⁻¹, HFC 2A vessels had a much smaller t50 of recovery compared with control vessels. In contrast, t50 of recovery was not different in 3A arterioles from HFC mice compared with control. The effect of NO blockade for both control and HFC mice is illustrated in Fig. 4 by the triangles (control: solid symbols, HFC: open symbols). With L-NNA, t50 of recovery was 10–15 s in each branch and for each condition; L-NNA reduced t50 of recovery predominantly in 2A arterioles of control mice.

Figure 4B shows relations between baseline diameter and baseline WSR (top) and dilation to end occlusion (middle) and peak RH (bottom). In each branch, baseline WSR was higher at smaller baseline diameters and was not different in HFC vessels compared with control vessel of comparable size. NO blockade resulted in a baseline vasoconstriction in vessels of control animals only, yet reduced baseline WSR in both control and HFC vessels. End occlusion dilation and peak reactive hyperemia dilation were also higher at smaller baseline diameters and not different in HFC vessels compared with control vessels of comparable diameter. L-NNA reduced peak RH dilation of 2A arterioles in control and HFC mice but did not affect dilation in 3A arterioles (Fig. 4B, bottom).

**DISCUSSION**

In the present study, we found evidence that atherogenic conditions result in an impairment of vasomotor control in 2A but not 3A arterioles. This impairment was concluded from the shorter duration of RH in 2A arterioles of HFC mice compared with control mice and was associated with a reduction in the NO contribution to baseline diameter and RH duration, as evidenced by NO blockade with L-NNA. Because the duration of RH increased with initial WSR upon flow restoration in 2A but not 3A arterioles, the susceptibility of 2A arterioles to impairment of endothelial function seems related to the sensitivity of this branch to shear stress. 2A arterioles therefore appear to represent the functional locus of hypercholesterolemia-associated microvascular dysfunction.

**Microvascular Responses During RH**

Among arterioles of different branch order as well as within each branch, we observed heterogeneity in 1) the magnitude of dilation during occlusion and RH and 2) the duration of the RH response (Fig. 2). Earlier studies in heart and striated muscle preparations have demonstrated heterogeneity in the RH response in microvessels as well as differences between 2A and 3A vessels (2, 9, 14, 22, 23, 34). The present study demonstrates that differences in shear stress sensitivity underlie branch differences in RH duration, whereas the heterogeneity in recovery times between vessels of the 2A branch are explained by differences in actual shear stress levels at the beginning of RH (Fig. 4A). In contrast, differences in baseline diameter underlie the heterogeneity in magnitude of dilation among branches and vessels (Fig. 4B). The greater dilations in small- versus large-size arterioles during occlusion and RH in the present and previous studies (14, 22, 34) seem explained by
the greater baseline vasomotor tone in the small versus large vessels, because basal vasomotor tone increases from proximal to distal vessels in the network (Fig. 1). Kanatsuka et al. (14) suggested also a relation between vessel diameter and duration of RH by demonstrating in in vivo canine hearts that vasodilation during RH was sustained for about two times longer in arterioles >100 μm than those smaller than this diameter. In the present study, we did not find a relation between $t_{50}$ of recovery and baseline diameter as such, but rather a difference in shear stress sensitivity between 2A and 3A branches. Thus, whereas $t_{50}$ increased with WSR at the beginning of RH in 2A arterioles, RH duration was independent of initial shear in 3A arterioles (Fig. 4A).

A shear stress effect in 2A arterioles seems consistent with the physiological role for this factor in NO release from the endothelium in the proximal part of the resistance vasculature...
(13, 19, 25). Indeed, NO blockade resulted in a greater baseline vasoconstriction in 2A than 3A arterioles in the present study. In addition, the l-NNA experiments demonstrated that the peak dilation and duration of the RH response were mediated by NO and that the NO contribution was pronounced in the 2A vessels (Fig. 4). However, as shown by the WSR dynamics in Fig. 3, bottom, the relation between shear stress and NO-mediated vasodilation during the maneuver of RH is not a straightforward one. Arteriolar WSR levels were actually diminished compared with baseline during RH except for the initial moments after release of the occlusion. We presumed that this step increase in WSR at begin RH is the major stimulus for endothelial NO release and affects arteriolar diameter throughout the RH recovery period in accordance with typical time delays of 3–15 s between an imposed change of flow and the observed diameter change (9, 16, 27). Besides determining the duration of RH, NO also contributed to peak RH dilation (Fig. 2). The involvement of NO in this part of the response seems too rapid to be explained by the shear stress increase at beginning of RH and might reflect NO release due to hypoxia (21, 24) or other mechanosensitive events (17). Because 3A arterioles in cremaster muscle have been demonstrated to dilate upon elevation of luminal shear stress by means of parallel occlusion (16), two explanations may account for the apparent low shear stress sensitivity of 3A compared with 2A arterioles during RH. First, because the WSR increase at the beginning of RH was on average lower than before occlusion in the 3A arterioles, the shear stress stimulus that prevailed upon flow restoration may have been too weak for significant NO release in these vessels. Second, a greater sensitivity of the smaller size 3A arterioles for myogenic vasoconstriction (6, 7), initiated by the increased luminal pressure upon release of the occlusion (21), might have overruled the NO-mediated dilator effect in these vessels. Hemodynamics at baseline as well as their alterations during the RH maneuver might well have been changed by the disruption of deferential vessels in the present experiments. Compared with the preparation with an intact cremaster-deferential microcirculation, total blood flow at baseline and during maximal vasodilation is reduced in the conventional open preparation that we used (11). Hill et al. (11) also showed that the deferential pathway was able to provide nearly sufficient collateral flow to maintain arteriolar blood flow in the cremaster when inflow was occluded. In our preparations, however, arteriolar blood flow was greatly reduced during the occlusion period, and as a result the metabolic stimulus for vasodilation might have been larger in our experiments as well.

**Impact of HFC on Arteriolar RH Response**

In comparison to control vessels, NO blockade did not affect baseline vasomotor tone and reduced the duration of RH to a much lesser extent in arterioles of HFC mice (Fig. 2). These observations therefore provide functional evidence for a diminished NO bioavailability (i.e., endothelial dysfunction) during atherogenic conditions. Because arterioles of ApoE3-Leiden and C57Bl/6 mice on the HFC diet demonstrated equally impaired responses (Fig. 2, B vs. C), the observed effects seemed due to the conditions of hyperlipidemia per se and not the result of genetic predisposition.

The ApoE3-Leiden mouse was shown before to be a useful model for study on the development and genetics of atherosclerosis (4, 10, 29–31). ApoE3-Leiden is one of the dysfunctional ApoE mutants that causes dysbetalipoproteinemia in humans because of an inefficient plasma clearance of ApoE-containing lipoprotein particles by the liver. ApoE3-Leiden mice express this human dysfunctional ApoE variant and have a similar dyslipidemia (29). To exacerbate the hypercholesterolemia, we placed the ApoE3-Leiden mice on a HFC diet: consistent with previous studies (30, 31), increases in plasma lipid concentrations were much more robust in transgenic animals compared with wild-type C57Bl/6 mice. This diet was further demonstrated to result in profound advanced atherosclerosis in the aorta of ApoE3-Leiden mice compared with C57Bl/6 mice on the same diet (10). Because vasomotor responses were not different between the HFC-fed groups in the present study, microvascular dysfunction and atherosclerosis of large conduit vessels seem not to develop in parallel upon induction of atherogenic conditions.

The microvascular impairment observed in HFC mice was confined to the endothelium, because the dilator response to SNP and adenosine was not changed (Fig. 1). The resistance vessels of the HFC mice developed normal basal vasomotor tone, but the contribution of NO to this basal tone was lost (Fig. 2, B and C). Despite this lack of NO, baseline shear rates were not decreased in HFC vessels (Fig. 4B), indicating that the microvascular network had adapted in such a manner that basal shear stress was maintained. Previous intravital studies in cremaster tissue similarly indicated an impairment of the endothelium and not of the smooth muscle cells during hypercholesterolemia and atherosclerosis (26, 35). These studies observed a ~50% reduction in dilation to acetylcholine in 3A arterioles: an equally impaired relaxation to bradykinin was found in 1A and 2A arterioles of HFC-fed ApoE3 mice in our laboratory (4). The current results indicate that the pathophysiological effects of the endothelial dysfunction are merely confined to 2A arterioles during RH (Fig. 4A). Moreover, the impairment of NO bioavailability seemed restricted to the shear stress-mediated component of NO release, because the NO contribution to peak RH dilation was not affected in HFC vessels (Figs. 2 and 4). In a parallel study (4) that was performed in our laboratory, the thickness of the glyocalyx in cremaster capillaries was found to be decreased in HFC mice. Because the glyocalyx is involved in the mechanosensing and transduction of shear stress to the endothelial cells (32), we hypothesize that degradation of the glyocalyx in the resistance vessels of HFC mice might underlie the decreased shear-dependent NO contribution that we observe in the present study. In conclusion, atherogenic conditions result in an impairment of vasomotor control during reactive hyperemia in shear stress-sensitive 2A arterioles. These vessels therefore appear to represent the functional locus of microvascular impairment associated with hypercholesterolemia and atherosclerosis.

**ACKNOWLEDGMENTS**

The authors thank Prof. Dr. L. M. Havekes for granting permission to use ApoE3-Leiden mice in the present experiments.

**GRANTS**

This study was supported by The Netherlands Organization for Scientific Research Grant 902-16-192 and a research fellowship from the Royal Netherlands Academy of Arts and Sciences (to H. Vink).
REFERENCES


