Effect of Steady Versus Oscillating Flow on Porcine Coronary Arterioles

Involvement of NO and Superoxide Anion

Oana Sorop, Jos A.E. Spaan, Terrence E. Sweeney, Ed VanBavel

Abstract—Coronary blood vessels are compressed by the contracting myocardium. This leads to oscillations in flow in the subendocardium. We examined the effects of steady and oscillating flow on isolated, cannulated subendocardial and subepicardial porcine arterioles. Steady flow–induced dilation in both vessel types, up to 12.9 ± 0.8% of the passive diameter in subendocardials and 9.6 ± 1.4% in subepicardials at 40 dyne/cm². Dilation was completely abolished after treatment with 10 μmol/L L-NNa. Sinusoidal modulation of steady flow at 1.5 Hz and 50% to 200% amplitude did not affect dilation. Oscillating flow without a net forward component with peak-peak shear values up to 100 dyne/cm² caused no dilation at all in these vessels. However, in the presence of 100 U/mL superoxide dismutase (SOD), oscillating flow induced dilation up to 19.5 ± 2.3% in subendocardial vessels and 11.5 ± 4.3% in subepicardials. LNNa (10 μmol/L) blocked this dilation by approximately 50%. SOD did not affect the magnitude of steady flow-induced dilation, but the response time after onset of steady flow shortened from 23.4 ± 1.5 to 14.3 ± 2.1 seconds. Diphenyleneiodinium, an inhibitor of NAD(P)H oxidase, uncovered dilation to oscillating flow in subendocardial vessels up to 9.5 ± 1.6%. Flow causes production of both NO and O₂⁻. During steady flow, the bioavailability of NO is sufficient to cause vasodilation. During oscillating flow, NO is quenched by the O₂⁻, suppressing vasodilation. Considering the pulsatile nature of subendocardial flow and the vulnerability of this layer, pharmacological manipulation of the balance between NO and O₂⁻ may improve subendocardial perfusion. (Circ Res. 2003;92:1344-1351.)

Key Words: flow-induced dilation ▪ coronary arterioles ▪ oxidative stress ▪ oscillating flow ▪ superoxide dismutase

Local coronary blood flow varies during the cardiac cycle. This is caused by the rhythmic generation of intramyocardial pressure¹ and elastance.² The resulting compression impedes perfusion during systole, when even retrograde flow may appear. The compression in the subendocardium contributes to the vulnerability of this layer to ischemia. The deleterious effects of this compression may be overcome by dilatory effects of pulsatile pressure and flow. Using isolated porcine coronary arterioles subjected to pulsatile transmural pressure³ at zero flow, we previously found vasodilation and increased sensitivity to vasodilators during pulsation compared with steady pressure. Variations in flow also could affect vascular tone. Many small vessels,⁴–¹⁵ including epicardial resistance vessels,⁸ show flow-dependent dilation to steady flow. Yet, little is known about the effect of pulsatile flow on these blood vessels. In addition, effects of flow on subendocardial vessels have not been studied at all.

Some evidence for contrasting responses to steady and pulsatile flow comes from studies on cultured endothelial cells.¹⁶ When these cells are exposed to oscillatory versus steady flow, lack of cell alignment in the flow direction,¹⁷ increased expression of adhesion molecules,¹⁸ lack of eNOS upregulation,¹⁹,²⁰ chronic increase in superoxide anion production,²¹ and different intracellular calcium transients²² are found. However, these differential effects of steady versus oscillating flow on endothelial biology cannot be extrapolated to vascular tone. Therefore, we aimed to directly assess the effect of steady versus oscillating flow on cannulated resistance arteries from the subendocardium (ENDO) and subepicardium (EPI). We show that steady but not oscillating flow caused dilation of ENDO and EPI vessels, which fully depended on NO. Addition of superoxide dismutase (SOD) or the NAD(P)H oxidase inhibitor diphenyleneiodinium (DPI) did not affect the response to steady flow but revealed a dilatory response to oscillating flow.

Materials and Methods

Surgery and Vessel Cannulation

All animal protocols were approved by the institutional laboratory animal care committee. Thirty-eight Yorkshire female pigs (H.W. Vendrig farm, Amsterdam, The Netherlands; 17 to 26 kg, 12 to 18
weeks old) were anesthetized by 4% halothane, ketamine (20 mg/kg), midazolam (1 mg/kg IM), and atropine (0.05 mg/kg). The animal was intubated and ventilated (O₂/N₂O, 1:2), the ear vein was cannulated, and midazolam (0.2 mg/kg) was administrated intravenously. After midsternal thoracotomy and heparinization (0.1 mL/kg, intravenously), the heart was fibrillated, excised, and immediately placed in cold (4°C) MOPS-buffered Ringer’s solution (in mmol/L: NaCl 145.0, KCl 4.7, MgSO₄ 1.17, CaCl₂ 2.0, NaH₂PO₄ 1.2, glucose 5.0, and pyruvate 2.0; the solution was equilibrated with air; pH 7.35±0.02). Dissection was performed in MOPS-buffered Ringer’s solution containing 1% albumin at 4°C. Subepicardial and subendo-cardial arterioles were dissected, cannulated at both ends with glass micropipettes, tested for leaks, and set to their in situ lengths.

Each cannula was supplied by a reservoir of MOPS buffer containing 1% bovine serum albumin. Each reservoir was separately pressurized by a computer-driven Venturi valve (FAIRCHILD T5200). Internal diameter of the vessel was continuously measured using a video technique. The vessel was superfused with MOPS buffer at a rate of 3 mL/min at 37°C. Drugs were added to the superfusion medium.

The vessels had internal diameters of 100 to 200 μm at full dilation and 60 mm Hg. Such vessels do not always develop substantial basal tone. In order to have a consistent tone level in all protocols, we preconstricted all vessels with 1° stress profiles, we first determined the required flow profiles, \( Q(t) \), from the following:

\[
Q(t) = \frac{\pi \cdot d^3}{32 \cdot \eta} \cdot \tau(t),
\]

with \( \tau(t) \) the shear stress, \( d \) the internal diameter after development of constant tone, and \( \eta \) the viscosity of 0.001 N·s/m². Then, the left and right pressure profiles needed for these values of flow at a constant intraluminal pressure of 60 mm Hg were determined from the following:

\[
P_l(t) = 60 + Q(t) \cdot R_l,
\]

\[
P_r(t) = 60 - Q(t) \cdot R_r,
\]

with \( R_l \) and \( R_r \) the left and right cannula resistances, as determined before start of the experiments. We used pipettes with matched resistances. Based on geometry, the resistance of the cannulated vessel segment was negligible compared with that of the cannulas.

Protocols

Experimental Group 1 (ENDO and EPI)

Steady flow (Figure 1 left traces), with shear between 1 and 90 dyne/cm², was achieved by a step change in cannula pressure from 60 mm Hg to the values calculated according to the above formulas. The different shear values were randomized. Flow was maintained for 3 minutes; flow steps were separated by 3-minute periods of zero flow. In some experiments, the pressure gradient, and consequently the flow through the vessels were reversed during the flow period. For purely sinusoidal oscillating flow without any net forward component (oscillating flow; Figure 1, middle), computer-generated sinusoidal pressure oscillations were applied to both cannulas. These oscillations, superimposed on 60 mm Hg, were in anti-phase and had a frequency of 1.5 Hz and amplitudes as calculated above. Peak-peak shear values of 1 to 100 dyne/cm² were applied in random order. Each oscillating shear level was applied for 3 minutes, separated by 3 minutes no-flow. Sinusoidal modulation of flow (“modulated flow”) was achieved by superimposing flow oscillations with a sine waveform at 1.5 Hz, on previously initiated net steady forward flow (Figure 1, right). The flow modulation amplitudes were 50%, 100%, 150%, and 200% of the steady flow value. For 100% modulation, flow just reached zero. Larger amplitudes include back flow, such as may occur in vivo during systole. These modulations were randomly applied on top of steady shear values of 10, 30, 60, and 100 dyne/cm² and maintained for 3 minutes, separated by 3 minutes steady flow.

Experimental Group 2 (ENDO and EPI)

To test the involvement of NO in flow-mediated dilation, steady flow steps were applied before and 30 to 40 minutes after the start of superfusion with 10 μmol/L L-NNA.
Group 3 (ENDO and EPI)
Steady and oscillating flow were applied before and 30 minutes after the start of superfusion with 100 U/mL superoxide dismutase (SOD).

Groups 4 and 5 (ENDO Vessels)
The mechanisms of oscillating flow-induced dilation in the presence of SOD were tested. After preconstriction, vessels were incubated in 100 U/mL SOD. Oscillating flow was applied. Subsequently, the vessels were incubated in either 10 μmol/L L-NNa as eNOS inhibitor or 80 U/mL catalase as H₂O₂ scavenger and the flow oscillations were repeated.

Group 6 (ENDO Vessels)
The effect of diphenyleneiodinium (DPI), a blocker of flavin-containing enzymes, such as NADP/H oxidase (possible sources of free radicals), was tested. Vessels were incubated with 30 μmol/L DPI for 30 minutes. Steady and oscillating flow protocols were applied before and during the incubation. Vessels in groups 3 to 6 were tested at 5 to 30 dyne/cm². These vessels were smaller than those in groups 1 to 2 and were found to be sensitive to lower shears.

Group 7 (Rat Cremaster Vessels)
In order to compare flow effects on vessels with preconstriction versus basal tone, we repeated the flow protocols of group 1 on comparably sized first order resistance arteries isolated from the rat cremaster muscle. These vessels develop stable, constant basal tone.

Accuracy of Flow Velocity Patterns
The accuracy of the experimental set-up in generating the desired levels of flow and shear was evaluated in separate experiments using an optical Doppler intravital velocimeter (Texas A&M University System Health Science Center). Red blood cell velocity was measured in two cannulated arterioles perfused with MOPS buffer containing rat erythrocytes. The measured centerline velocity matched the value calculated on the basis of cannula resistances, left and right cannula pressures, vessel diameter, and Poiseuille flow within 10%. This was the case for steady flow as well as for oscillating and modulated flow with frequencies between 0.5 and 2 Hz (online Figure 1). In order to estimate whether the velocity profile in the vessel was fully parabolic during oscillating flow, we calculated the Womersley number (WO). When WO exceeds unity, the velocity profile is no longer parabolic, and the flow is phase-shifted in time relative to the oscillating pressure gradient.23 WO in our experiments did not exceed 0.01, indicating quasi-steady behavior of the fluid with a parabolic flow profile during all protocols.

Drugs
Bradykinin, U46619, L-NNa, SOD S-2515, Catalase, and DPI were purchased from Sigma Chemicals.

Data Analysis
All vascular diameters as well as the degree of dilation were normalized to maximum dilated diameters of the vessels, as obtained in response to 0.1 μmol/L bradykinin at 60 mm Hg intraluminal pressure. For each 3-minute flow intervention, the time-averaged diameter was obtained after development of a stable response. Data are presented as mean ± SEM. Data were analyzed by one-way and two-way ANOVA, including the interaction term followed by Bonferroni post hoc tests where appropriate. Differences were considered significant for P < 0.05.

Results
Effects of the various flow patterns were tested in 7 ENDO (159 ± 15 μm inner diameter) and 6 EPI (163 ± 18 μm inner diameter) arterioles (experimental group 1). In response to a sudden onset of steady flow, all vessels developed flow-induced dilation. Figure 1 (left) depicts a typical example. Dilation developed after a lag time of 15 to 25 seconds from the flow onset, and reached a plateau value in approximately 1 minute. This value of dilation remained constant for as long as flow was maintained. After stopping flow, the vessel regained its initial level of tone within 2 minutes. The middle panels of Figure 1 depict the response of this vessel to oscillating flow. In this example, flow-dependent dilation did not occur in response to an oscillating shear stress of 10 dyne/cm² at 1.5 Hz. The right panels indicate that 100% modulation of 10 dyne/cm² steady flow did not induce extra vasodilation.

Figures 2 and 3 summarize the effects of the various flow patterns on tone. For steady flow (Figure 2A), dilation in ENDO and EPI vessels became significant at 10 dyne/cm². Dilation became larger with increasing shear stress up to approximately 40 dyne/cm² and tended to decrease again at still higher shear values. Dilation was significantly larger in ENDO as compared with EPI vessels (P < 0.05, two-way ANOVA).

A lack of dilation in response to oscillating flow was found in all vessels; the diameter of some vessels remained unchanged, whereas others (both ENDO and EPI) showed a small constriction. Figure 2B presents the average results of experiments performed on 5 ENDO and 6 EPI arterioles. Oscillating flow at peak-peak shear levels up to 100 dyne/cm²
did not significantly change the vessel tone ($P$=NS, two-way ANOVA). In 2 tested vessels, oscillating flow profiles at lower (down to 0.1 Hz) and higher (2 Hz) frequencies and similar shear values also did not induce flow-dependent dilation (data not shown).

The effects of flow modulation on 7 ENDO and 6 EPI arterioles are depicted in Figure 3. Modulating shears of 10 and 30 dyne/cm$^2$ at 1.5 Hz did not significantly change the level of flow-induced dilation ($P$=NS, two-way ANOVA). Even at amplitudes of 150% and 200%, when retrograde flow occurred, and the root-mean-square level of shear stress was increased by respectively 46% and 73% as compared with steady flow, the diameter remained constant during the 3 minutes of flow modulation. This was the case for both ENDO and EPI arterioles. Modulation of shear values around averages of 60 and 100 dyne/cm$^2$ up to 200% also did not cause additional dilation (data not shown).

The order of the steady, oscillating, and modulated flow protocols was randomized. In those cases where steady flow was applied after the other protocols, normal dilation occurred. Sensitivity to bradykinin was tested in all vessels after finishing all protocols and found to be normal, indicating undamaged endothelial cells (data not shown).

In 10 vessels (5 ENDO, 5 EPI), the effect of L-NNA was tested. In this group, shear-induced dilation before L-NNA was significant above 5 dyne/cm$^2$. In ENDO and EPI vessels, incubation with 10 μmol/L L-NNA in the absence of flow for 30 minutes induced a 3% to 4% constriction. L-NNA completely abolished dilation over the whole range of shear stresses in ENDO and EPI vessels (Figure 4; $P$<0.05, two-way ANOVA).

In 7 ENDO (127±10-μm inner diameter) and 6 EPI (132±12-μm inner diameter), steady and oscillating flow protocols with shears up to 30 dyne/cm$^2$ were applied before and after incubation for 30 minutes with 100 U/mL SOD. SOD had no effect on the basal diameter of the vessels. Figures 5A and 5B show that the magnitude of the response to steady flow remained unchanged ($P$=NS, two-way ANOVA). Whereas oscillating flow in the absence of SOD had no effect on vessel diameter, Figures 5C and 5D show that after treatment with SOD such oscillating flow caused significant dilation in all vessels ($P$<0.05, 2-way ANOVA; eg, at 30 dyne/cm$^2$: 0.0±1.2% versus 19.5±2.3% in ENDO and 0.0±1.1% versus 11.5±4.3% in EPI vessels, before versus after incubation). In ENDO vessels in the presence of SOD, the response to oscillating flow at 30 dyne/cm$^2$ peak-peak even exceeded the dilation to steady flow ($P$<0.05, paired $t$ test, n=7).

Although SOD did not affect the magnitude of dilation to steady shear, it did affect the latency of the dilatory response (Figure 6). We found a 21.8±2.7-second delay to 25% of the dilatory response after onset of steady flow, which was independent of the level of shear (Figure 6A; $P$=NS; n=7 ENDO+6 EPI). After incubation with SOD, the delay time was reduced from 23.4±1.5 to 14.3±2.1 seconds at 10 dyne/cm$^2$ (Figure 6B, n=5; $P$<0.05, one-way ANOVA). In the presence of SOD, the delay time for start of dilation after onset of oscillating flow was slightly larger than for steady flow (16.3±1.8 second; $P$=0.044).

We tested in 4 ENDO vessels (128.5±11.2 μm) whether NO mediates oscillating flow-induced dilation in presence of
SOD. Oscillating flow with peak-peak shears up to 30 dyne/cm² was applied. L-NNA (10 μmol/L) reduced the dilation in the presence of SOD by approximately 50% (Figure 7A; P<0.05, two-way ANOVA). The remaining dilation could not be attributed to an H₂O₂ mediated mechanism, because in 5 ENDO vessels (134.3±12.1-μm inner diameter), 80 U/mL catalase did not significantly diminish the SOD effect during oscillating flow (Figure 7B; P=NS, SOD versus SOD+catalase). In two vessels tested, this concentration of catalase reduced the dilation to 50.7±10.0% from 50.7±10.0% to 8.8±1.0%. In other experiments, raising the catalase concentration from 80 to 1000 U/mL only modestly impaired oscillating flow-induced dilation: from 9.1±1.0% (SOD) to 7.3±1.0% (SOD+1000 U/mL catalase) at 10 dyne/cm² (n=2), and from 18.4% to 16.5% at 30 dyne/cm² (n=1).

Five ENDO arterioles (129.7±6.8 μm) were incubated with 30 μmol/L of the NAD(P)H oxidase inhibitor DPI. The dilation to oscillating flow was recorded for peak-peak shears of 5 to 30 dyne/cm². Oscillating flow-induced dilation was absent before incubation with DPI, but became significant at 10 dyne/cm² in the presence of DPI (Figure 7C). The dilation was approximately 20% to 30% less in the presence of DPI as compared with SOD, as seen in Figures 5C, 7A, and 7B. Indeed, in 3 of these 5 vessels, we also tested the effect of SOD and found it to be significantly larger. DPI did not affect steady flow-induced dilation.

The above effects on coronary vessels were obtained after preconstriction by U46619. We tested in 9 first order rat cremaster arterioles (159±19 μm) whether vessels with basal tone also lack responsiveness to oscillating flow. Basal tone developed to approximately 65% of the maximal diameter. Figure 8 indicates that steady flow caused dilation above 25 dyne/cm². Furthermore, sinusoidal modulation of flow at 100% did not affect the dilation in also these vessels. However, also in these vessels, dilation to oscillating flow at 1.5 Hz was absent up to peak-peak amplitudes of 90 dyne/cm² (P=NS, steady versus modulated flow; P<0.01 steady versus oscillating; P=NS, oscillating flow versus no flow, ANOVAs). The effect of SOD was not tested in these vessels with basal tone. We were able to cannulate 3 ENDO vessels smaller than 100 μm, which developed basal tone that averaged 56.6±0.9% from the maximal dilated diameter. In these vessels, SOD incubation also uncovered oscillating flow-induced dilation of 18.8±2.6% of maximal diameter at a peak-peak shear of 30 dyne/cm².

**Discussion**

This study presents several novel findings. First, dilation in response to steady flow occurred not only in epicardial arterioles but also in endocardial vessels, which responded to a greater degree than did the epicardial vessels. The dilation was NO dependent, being completely blocked by L-NNA. Second, oscillating flow without a net forward component did not cause flow-dependent dilation. However, in both EPI and ENDO vessels, dilation to oscillating flow did occur after incubation with superoxide dismutase. In fact, in the presence of SOD, ENDO vessels dilated to a greater degree in response to oscillating flow than to steady flow. This dilation was partially mediated by NO; the remaining dilation occurred via a mechanism not related to H₂O₂, because catalase was without effect. Third, DPI, an NAD(P)H oxidase inhibitor, also uncovered a dilation to oscillating flow in ENDO vessels. Fourth, the delay time for steady flow-induced dilation decreased in the presence of SOD in both ENDO and EPI vessels.
The possibility to independently manipulate left and right cannula pressure by computers allowed us to apply flow wave-forms as would occur in the beating myocardium at constant intraluminal pressure. We are confident that luminal pressure was constant, capacitive flow was absent, and flow was accurately predicted from cannula pressures and cannula resistances in the current study for the following reasons: first, a sudden or oscillating change in luminal pressure would have resulted in simultaneous distension of the vessel. Previous studies demonstrated that pressure variations of less than 5 mm Hg can be detected in this way. No such changes in diameter were observed even though gradients in cannula pressures in the order of 100 mm Hg were required to generate the highest shear stresses. Second, in previous experiments at zero flow, intraluminal pressure as measured by servo-null pipettes penetrated through the vessel wall matched the cannula pressures, also for fast oscillations at high amplitudes. Third, the low estimated Womersley number (see Materials and Methods) indicates a fully developed parabolic velocity profile that instantaneously followed the applied pressure gradient. Fourth, we found the predicted centerline velocity to match the velocity of red blood cells in separate experiments.

Basal tone in porcine coronary vessels of the size we studied is variable and generally quite shallow. Therefore, we preconstricted vessels by U46619. We do not believe that this preconstrictor is involved in the diverging effects of steady and oscillating flow, because the cremaster arterioles, as well as the few coronary arterioles below 100 μm that we were able to cannulate, which did have basal tone, responded in the same way to the various flow patterns. The applied shears included clearly superphysiological levels, in order to dem-
onstrate absence of oscillating flow-induced dilation over a wide range. We do not believe that the lower shear respon-siveness of EPI vessels relates to damage during dissection because similar levels of preconstriction were obtained by 1 μmol/L U46619 in ENDO and EPI vessels and our previous studies \(^3\) showed similar concentration-response curves to adenosine and bradykinin in both vessel types.

In the first experimental group of vessels studied, having diameter around 150 to 180 μm, we found a half-maximal effect of shear at approximately 20 dyne/cm\(^2\) (Figure 2). Smaller arterioles (110 to 150 μm) were used in most of the other experiments. In these vessels the dilation reached a plateau at shears of 10 dyne/cm\(^2\). The observation that the smaller vessels are more sensitive to shear is in accordance with data of Kuo et al \(^9\) on epicardial arterioles, showing that shear sensitivity is the largest in 89-μm vessels, whereas larger vessels were less sensitive to flow.

**NO and O\(_2^-\) Production**

It is now accepted that NO bioavailability within the vascular wall is negatively influenced by O\(_2^-\) production. \(^{24}\) Both radicals react extremely rapidly to form the ONOO\(^-\) (peroxynitrite) anion. Their balance therefore is of major impor-
tance in vascular function. Our experiments indicate that steady and oscillating flow differentially affect this balance. We suggest that steady flow induced production of NO that overwhelmed any possible concomitant O\(_2^-\) production, resulting in NO-dependent dilation. In contrast, during oscillating flow, O\(_2^-\) production fully inhibited bioavailability of the simultaneously produced NO to the smooth muscle cells. In the presence of extrinsic SOD or DPI, this inhibition was reversed, resulting in oscillating flow-induced dilation. It is not clear whether the negative balance during oscillating flow is due to a higher O\(_2^-\) production or a lower NO production.

Modulation of steady flow up to 200% (Figure 3) neither reversed nor augmented the dilation. There are alternative explanations for this finding. Thus, the effects of any putative extra NO and O\(_2^-\) production during such modulation could have just canceled. Alternatively, the effect of dynamic flow components may be different, depending on the presence or absence of a net forward flow. Thus, superoxide production by rhythmic flow may have been suppressed by the presence of a net forward flow. However, it is not clear then why even a 200% modulation of low shear (eg, changing from a steady 10 dyne/cm\(^2\) to shear oscillations between -10 and 30 dyne/cm\(^2\); Figure 3A) did not augment the initial dilation (which was not yet saturated; compare Figure 3B), whereas oscillations with such amplitudes had clear effects after removal of the superoxide (Figure 5, right).

In ENDO vessels at 30 dyne/cm\(^2\), the response to oscillating flow in the presence of SOD was even larger than that to steady flow. In addition, part of the former response could not be inhibited by L-NNA or catalase. This suggests the existence of still another factor produced by oscillating but not steady flow. Whatever the nature of this factor, it is sensitive to O\(_2^-\) because no oscillating flow-induced dilation at all was observed in the absence of SOD. Recently, it was shown that H\(_2\)O\(_2\) is a possible EDHF in human coronary microvessels. \(^{25}\) One could thus argue that the uncovered dilation to oscillating flow in the presence of SOD relates to H\(_2\)O\(_2\). However, 80 or 1000 U/mL catalase was without effect, whereas 80 U/mL almost fully suppressed the dilation to extrinsic H\(_2\)O\(_2\).

We do not believe that steady flow, once developed, caused large amounts of O\(_2^-\) production, because SOD was without any effect on the magnitude of steady flow-induced dilation. However, the onset of steady flow represents a transient by itself, and this might induce O\(_2^-\) production. There is a very consistent lag time that precedes the initiation of vasodilation on onset of flow. The duration of the delay ranges between 5 seconds in arterioles smaller than 100 μm\(^4\) to 40 seconds in large conduit arteries. \(^{26,27}\) In the present study, we found a 20-second delay. On application of SOD, a 40% reduction in delay time was observed, supporting the view that the onset caused O\(_2^-\) production. The remaining delay was similar for onset of steady and oscillating flow, suggesting that similar mechanisms caused the dilation. Indeed, L-NNA also substantially inhibited the oscillating flow-induced dilation in the presence of SOD, indicating the involvement of NO also here.

It was beyond the scope of this article to unambiguously identify the source of O\(_2^-\) production during oscillating flow or quantify its production. Experiments on cultured endothelial cells that demonstrate superoxide production in response to oscillating shear support our SOD and DPI data, which together implicate NAD(P)H oxidase as a shear-induced O\(_2^-\) source. Alternative sources may include xanthine oxidase, cytochrome P450, or eNOS itself. In addition to its effect on NAD(P)H oxidase, DPI may inhibit other flavin-containing oxidases, including eNOS. Therefore, we cannot fully rule out that eNOS could have contributed as an additional source of oxygen-free radicals. However, this does not affect our main thesis that superoxide production during oscillating flow inactivates nitric oxide.

Using dihydroethidine (DHE), we attempted to perform dynamic measurements of O\(_2^-\) production in our cannulated vessels during acute episodes of shear. We now believe that this approach was unsuccessful because of a number of complicating factors, including the effect of flow on the transport rate of the dye to the cells of interest. Tyrosine nitrosylation neither seemed a way to directly demonstrate O\(_2^-\) and subsequent ONOO\(^-\) because these nitrosylation reactions require chronic models or at least substantial stim-ulus periods and are therefore not applicable to acute inter-
ventions such as studied here. Cytochrome c reduction has been applied for the measurement of O\(_2^-\). However, this absorbance technique seems not sensitive enough to use on perfused arterioles. Although our data strongly suggest a role for O\(_2^-\) in the effects we observed, future studies must be directed at online, localized quantification of O\(_2^-\) production during episodes of acute shear.

**Implications for Coronary Physiology**

This study demonstrates that not only EPI \(^b\) but also ENDO arterioles show steady flow-induced dilation. Moreover, we found such dilation to fully depend on NO-dependent path-
ways. Whether these responses are relevant for coronary flow regulation depends on in vivo shear stresses in the coronary circulation. These are poorly defined especially in the suben-
Oscillating Flow Effects in Coronary Arterioles

Oana Sorop was supported by grant 98.180 of the Netherlands Heart Foundation, Terry Sweeney was supported by the University of Scranton, the Netherlands Organization for Scientific Research grant 1F33 HL072548-01. Sorop et al measured microvascular diameters and velocities in canine epicardial vessels. Basal shear stress averaged 10 dyne/cm² in small arteries and ranged between 15 and 23 dyne/cm² in vessels of 100 to 150 μm. This coincides with the range of values causing submaximal dilation in EPI vessels of this size. A concern however is that smaller vessels respond to lower shears, as demonstrated by Kuo et al and confirmed in the present study, whereas based on coronary branching patterns, the in vivo shear stress on average actually increases in smaller vessels.

The dynamic profiles of flow and shear along the coronary microcirculation are not extensively quantitated. However, based on the retrograde coronary flow in systole and subendocardial velocity and diameter patterns, the highly pulsatile nature of subendocardial flow is beyond doubt. Our modulated flow data (Figure 3) make clear that in vitro such pulsatility does not add another component to flow-dependent dilation. In that sense, the pulsatility seems irrelevant for subendocardial flow regulation. However, the activity in vivo of eNOS, O₂⁻-producing enzymes, and also intrinsic SODs (notably extracellular SOD3) may differ from that observed in vitro. Moreover, their activity is influenced by coronary artery disease. The diverging actions of purely oscillating and steady flow on the NO-O₂⁻ balance therefore leave room for quite substantial effects of flow modulation under such conditions.

In conclusion, our data show that steady and oscillating flow differentially modulate coronary vascular tone via mechanisms that determine the balance between NO and O₂⁻ production in the coronary circulation. Considering the pulsatile nature of subendocardial flow and the vulnerability of this layer, interfering with this balance may provide future means for improving subendocardial perfusion.

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Online Figure 1: experiments validating the estimation of shear stress. Top: validation for two vessels at steady shear. The x-axis shows shear stress as estimated from the applied pressure gradients, cannula resistances and vascular diameter; the y-axes indicate the shear stress based on the measured centerline velocity of a few red blood cells in the perfusate, on the basis of a parabolic velocity profile. The identity line (x=y) is shown. Middle: Estimated and measured peak-peak shear stress during sinusoidal (1.5 Hz) oscillating flow without a net forward component. This plot indicates that flow oscillations linearly follow pressure oscillations, without any damping at this frequency. Bottom: Effect of modulating flow up to 200% on the average shear stress as determined from centerline velocity. Pressure gradients were applied that should induce variations around an average level of 30 dynes/cm². The time-averaged shear stress based on red blood cell velocity was close to this value. This figure indicates that large variations in driving pressure do not cause unintended shifts in the average shear stress.

Online Figure 2: response to very low shear stress in 6 sub-endocardial arterioles, 123 ± 12 (SEM) μm in diameter, preconstricted by 1 μM U46619. In order to be able to apply this low shear stress to the small vessels with sufficient accuracy, these vessels were cannulated with pipettes having higher resistances than used in the main study.
Steady flow

Estimated shear (dynes/cm²)

Measured shear (dynes/cm²)

Oscillating flow

Estimated shear (dynes/cm²)

Measured shear (dynes/cm²)

Modulated flow of 30 dynes/cm²

Pulsatility (%)
Normalized dilation