Cytosolic Alkalinization of Vascular Endothelial Cells Produced by an Abrupt Reduction in Fluid Shear Stress

Roy C. Ziegelstein, Paul S. Blank, Linda Cheng, Maurizio C. Capogrossi

Abstract—Reductions in fluid shear stress produce endothelium-dependent vasoconstriction and promote neointimal hyperplasia, but the intracellular signaling mechanisms involved in these processes are poorly understood. To examine whether decreases in fluid shear stress affect endothelial cytosolic pH, carboxy-seminaphthorhodafluor-1–loaded rat aortic endothelial cells were cultured in glass microcapillary tubes and examined during abrupt reductions in laminar flow. After a 30-minute exposure to a shear stress of 2.7 dyne/cm² in bicarbonate buffer, the acute reduction of fluid shear stress from 2.7 to 0.3 dyne/cm² transiently increased cytosolic pH from 7.20±0.02 to 7.47±0.07 (mean±SEM, P<.05 versus control). This was not affected by prior inhibition of the Na⁺-H⁺ exchanger with 10 μmol/L ethylisopropylamiloride but was abolished in bicarbonate-free buffer. Recovery from an ammonium chloride prepulse–induced acid load occurred more rapidly when fluid shear stress was abruptly reduced from 2.7 to 0.3 dyne/cm² after maximal acidification (+0.04±0.02 pH unit at 2 minutes) than when shear stress was maintained at 2.7 dyne/cm² continuously (0.00±0.00 pH unit at 2 minutes, P<.05). This accelerated cytosolic pH recovery was dependent on the presence of bicarbonate ion and was blocked by the addition of the exchange inhibitors DIDS (100 μmol/L) and ethylisopropylamiloride or by removal of buffer Na⁺, indicating that the acute reduction in fluid shear stress activates the extracellular Na⁺–dependent Cl⁻-HCO₃⁻ exchanger and the Na⁺-H⁺ exchanger and increases cytosolic pH in vascular endothelial cells. (Circ Res. 1998;82:803-809.)

Key Words: endothelium ♦ cytosolic pH ♦ shear stress ♦ carboxy-seminaphthorhodafluor-1 ♦ DIDS

The vascular endothelium responds to increases in fluid shear stress with changes in intracellular signal transduction pathways that begin within seconds of the initiation of the mechanical stimulus.1 Among these rapid shear stress responses are membrane hyperpolarization,2 inositol trisphosphate generation,3 and cytosolic acidification.4 If the stimulus is sustained, alterations in gene expression4–8 and reorganization of the endothelial actin cytoskeleton then take place over hours.9,10

When endothelial cells cultured in microcapillary tubes are exposed for brief periods (2 to 5 minutes) to an abrupt increase in fluid shear stress from 0.3 to 13.4 dyne/cm² in a bicarbonate buffer, pHᵢ decreases by 0.09 pH unit and then returns to baseline after flow is reduced back to control levels.4 This intracellular acidification is sustained during a 30-minute exposure to shear stresses of 13.4 dyne/cm², although partial recovery is observed over this same time period at lower shear stresses. The magnitude of the pHᵢ change is dependent on the change in shear stress, with a threshold below 0.5 dyne/cm² and a maximal effect between 6.7 and 13.4 dyne/cm².4 The decrease in pHᵢ produced by an increase in fluid shear stress occurs within seconds after an abrupt increase in flow in both rat1 and bovine3 aortic endothelial cells and is due to the net effect of activation of both an alkali exchanger, extracellular Na⁺–independent Cl⁻-HCO₃⁻ exchange, and an acid exchanger, Na⁺-H⁺ exchange.4

Acute reductions in fluid shear stress are associated with endothelium-dependent vasoconstriction11 and with proliferation of subjacent neointimal smooth muscle cells, which may be stimulated by growth factors derived from the endothelium.12 Areas of rapidly decreasing fluid shear stress and of flow reversal occur near arterial branches, sites prone to the development of atherosclerotic plaque.13–15 These findings suggest that reductions in fluid shear stress may activate signal transduction mechanisms that alter vascular reactivity and, if sustained, may stimulate atherogenesis. The present study was performed to determine whether an abrupt reduction in fluid shear stress affects endothelial pHᵢ.

Materials and Methods

Cell Culture

Endothelial cells were cultured from the descending thoracic aortas of 2- to 4-month-old Wistar rats by the primary explant technique.5,16

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Rat aortic endothelial monolayers were grown to passages 2 to 14 in minimum essential medium with d-valine supplemented with 10% fetal calf serum, 100 µg/mL endothelial mitogen, 5 mg/mL penicillin, 5 mg/mL streptomycin, and 10 mg/mL neomycin (GIBCO) at 37°C in a humidified atmosphere of 95% air/5% CO₂. Endothelial cells were identified by demonstrating specific immunofluorescent staining for factor VIII–related antigen (DAKO Corp). After treatment with 0.25% trypsin and 0.5 mmol/L EGTA (Sigma Chemical Co), cells were plated in 1-mm² glass capillary tubes (Vitro Dynamics) precoated with 1% gelatin (Sigma) and allowed to grow to confluence on one face of the tube for 24 to 48 hours before experimental use.

Measurement of Cytosolic pH

Endothelial monolayers were loaded with the membrane-permeant ester derivative of the pH-sensitive fluorescent probe c-SNARF-1 (c-SNARF-1/AM, Molecular Probes) as previously described. Briefly, cells were loaded with 3–5 µmol/L c-SNARF-1/AM in culture medium in a 95% air/5% CO₂ incubator at room temperature for 30 minutes. They were then washed in indicator-free buffer for an additional 60 minutes before experimental use. After excitation at 530±5 nm on the stage of a modified inverted microscope, the 590±5-nm/640±5-nm ratio of emitted fluorescence was used to measure pH. A pH calibration was obtained from c-SNARF-1–loaded endothelial monolayers exposed to solutions of varying pH values containing 140 mmol/L KCl, 20 µmol/L nigericin, 1 µmol/L valinomycin, and 1 µmol/L carbonyl cyanide p-(trifluoromethoxy)-phenylhydrazone at 23°C. Under these loading conditions, c-SNARF-1 fluorescence localizes exclusively in the cytosol of rat aortic endothelial cells.

Experimental Protocols

The glass capillary mounted on the stage of the modified inverted microscope was internally perfused with a nonpulsatile, integrated-drive, positive-displacement pump (Cole-Parmer), achieving steady laminar flow. For some experiments, a bicarbonate solution of the following composition was used (mmol/L): NaCl 116.4, KCl 4.9, MgSO₄ 1.2, NaHCO₃ 26.2, NaH₂PO₄ 1.0, D-glucose 5.6, and CaCl₂ 1.5; this was continuously gassed with 95% O₂/5% CO₂ to maintain pH at 7.38±0.02. In other experiments, the exchangers were inhibited by replacing buffer Na⁺ by equimolar choline.

Data Analysis

Data are presented as the mean±SE. When comparing the experimental results with control within a monolayer, a Student’s t test for paired analysis was used. When comparing different monolayers, a Student’s t test for unpaired variables was performed. Repeated-measures analysis with polynomial contrasts was used to examine time interaction terms between groups.

Results

Effect of an Abrupt Decrease in Fluid Shear Stress on Endothelial pH

To determine the effect of an abrupt decrease in fluid shear stress on endothelial pH, endothelial monolayers were first exposed to an increase in shear stress from 0.3 to 2.7 dyne/cm² for 30 minutes in bicarbonate buffer solution, and then shear stress was abruptly reduced again to 0.3 dyne/cm² (Figure 1A). When shear stress was increased, pH decreased from 7.22±0.03 to 7.12±0.03 (n=12, P<.01). During the period of continuous fluid shear stress at 2.7 dyne/cm², pH gradually recovered to 7.20±0.02 by the end of the 30-minute exposure. When fluid shear stress was then abruptly reduced from 2.7 to 0.3 dyne/cm², pH increased above baseline to 7.47±0.07 (P<.05 versus control) before returning to control pH levels over 15 to 20 minutes. In bicarbonate-free (HEPES) buffer, increasing fluid shear stress from 0.3 to 2.7 dyne/cm² resulted in a small increase in pH, as previously described. When fluid shear stress was abruptly reduced from 2.7 to 0.3 dyne/cm² in bicarbonate-free solution, no cytosolic alkalinization was observed (Figure 1B).

Effect of Na⁺-H⁺ Exchange Inhibition on the Cytosolic Alkalinization During an Abrupt Reduction in Fluid Shear Stress

To determine the effect of Na⁺-H⁺ exchange inhibition on the increase in pH during an abrupt reduction in fluid shear stress, monolayers were pretreated with the Na⁺-H⁺ exchange inhibitor EIPA (10 µmol/L). This concentration of EIPA completely abolished pH recovery from an NH₄Cl pulse.
acidification in bicarbonate-free buffer (data not shown). The Na\(^+\)-H\(^+\) exchanger may be inhibited by EIPA before shear stress exposure without affecting the flow-dependent decrease in pHi, since this is primarily due to activation of the extracellular Na\(^+\)–independent Cl\(^-\)-HCO\(_3^−\) exchanger. As shown in Figure 2, EIPA did not block the alkalinization after shear stress exposure in bicarbonate-containing solution (n=4, P=NS versus control). Thus, the cytosolic alkalinization during an abrupt reduction in fluid shear stress is not due to activation of Na\(^+\)-H\(^+\) exchange.

Effect of an Abrupt Decrease in Fluid Shear Stress on Recovery From Intracellular Acidification

To further examine the mechanism of acid extrusion stimulated by an abrupt reduction in fluid shear stress, flow was abruptly reduced during recovery from an intracellular acid load imposed by an NH\(_4\)Cl prepulse. During continuous shear stress at 2.7 dyne/cm\(^2\), endothelial pHi responded in a predictable fashion to a brief exposure to NH\(_4\)Cl and washout in a bicarbonate buffer equilibrated with 5% CO\(_2\) (Figure 3A). In the presence of NH\(_4\)Cl, pHi rapidly increased from a baseline of 7.11±0.03 to 7.26±0.03 (n=38) as a result of the rapid diffusion of NH\(_3\) into the cells and its combination with protons. The pHi then gradually began to recover in the continued presence of NH\(_4\)Cl as a result of the diffusion of NH\(_4^+\) into cells and its dissociation into NH\(_3\) and H\(^+\). When monolayers were returned to bicarbonate buffer without NH\(_4\)Cl, an intracellular acidification occurred (minimum pHi 7.04±0.02) that was due to the excess of H\(^+\) left when NH\(_3\) rapidly exits the cells on washout of NH\(_4\)Cl. The pHi recovery from this acid load then occurred slowly over 15 to 20 minutes. Recovery from an acid load may proceed via activation of Na\(^+\)-H\(^+\) exchange (inhibited by EIPA) and, in some cell types, via an extracellular Na\(^+\)–dependent Cl\(^-\)-HCO\(_3^−\) exchanger, which regulates pHi by extruding acid in physiological bicarbonate solution. This exchanger is irreversibly inhibited after pretreatment with the anion exchange inhibitor DIDS. The pHi of monolayers pretreated with the combination of EIPA and DIDS was 6.80±0.03. When exposed to NH\(_4\)Cl, pHi increased to 7.01±0.01 (Figure 3B) and then decreased rapidly to 6.69±0.04 when monolayers were returned to buffer without NH\(_4\)Cl. Recovery from the NH\(_4\)Cl prepulse acid load was abolished by the combination of DIDS and EIPA during continuous exposure to fluid shear.

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**Figure 2.** Effect of Na\(^+\)-H\(^+\) exchange inhibition on the change in endothelial pHi during an abrupt reduction in shear stress. Recordings show representative c-SNARF-1 fluorescence from a rat aortic endothelial monolayer exposed to abrupt changes in shear stress under control conditions (left) and in the presence of the Na\(^+\)-H\(^+\) exchange inhibitor EIPA (right). The monolayer was first exposed to an increase in fluid shear stress from 0.3 to 2.7 dyne/cm\(^2\) for 30 minutes in bicarbonate buffer. After the 30-minute period at a shear stress of 2.7 dyne/cm\(^2\), shear stress was abruptly decreased to 0.3 dyne/cm\(^2\). After the initial recording (left), the buffer was changed to an identical bicarbonate solution with 10 μmol/L EIPA. The increase in pHi during an abrupt reduction in fluid shear stress was not affected by EIPA (n=4, P=NS).

**Figure 3.** Effect of NH\(_4\)Cl on endothelial pHi. A, Recording shows representative c-SNARF-1 fluorescence from an endothelial monolayer exposed sequentially to 20 mmol/L NH\(_4\)Cl in bicarbonate buffer for 4 minutes followed by washout (n=38). Cells were acid-loaded by applying and then withdrawing NH\(_4\)Cl (20 mmol/L NH\(_4\)Cl replaced 20 mmol/L NaCl in the standard bicarbonate solution) at a constant shear stress of 2.7 dyne/cm\(^2\). Recovery from the acid load proceeded slowly over 15 to 20 minutes. B, When cells were incubated with 100 μmol/L DIDS and 10 μmol/L EIPA for 1 hour before NH\(_4\)Cl exposure (n=5), baseline pHi was lower than under control conditions because of the combined inhibition of extracellular Na\(^+\)–dependent Cl\(^-\)-HCO\(_3^−\) exchange and Na\(^+\)-H\(^+\) exchange. Recovery from the acid load during continuous shear stress of 2.7 dyne/cm\(^2\) was blocked by the combination of these inhibitors.
stress of 2.7 dyne/cm² (pH i 6.70 ± 0.02 at 15 minutes, n=3, P=NS versus the minimum pH i after NH₄Cl washout).

In other experiments, endothelial monolayers were exposed to an NH₄Cl pulse at a shear stress of 2.7 dyne/cm² after a period of at least 30 minutes of continuous shear stress at the same flow rate. At the point of maximal acidification during washout of NH₄Cl (determined by viewing the display of measured fluorescence simultaneously on a computer screen), fluid shear stress was abruptly reduced from 2.7 to 0.3 dyne/cm², and the rate of recovery from the intracellular acid load under these conditions was compared with the rate of recovery at a continuous shear stress of 2.7 dyne/cm². During continuous shear stress at 2.7 dyne/cm² (Figure 4A), there was no recovery from the NH₄Cl-induced acid load during the first 2 minutes after maximum acidification (ΔpH i 0.00 ± 0.00, n=19; only data paired with an NH₄Cl-induced acid load in which flow was reduced during recovery are included). B, When fluid shear stress was abruptly reduced from 2.7 to 0.3 dyne/cm² during recovery from the acid load, pH i increased by 0.04 ± 0.02 at 2 minutes (P<.05 vs control, n=19). C, The increased early recovery during abrupt reduction in fluid shear stress was partially inhibited by the combination of DIDS and EIPA (ΔpH i 0.02 ± 0.01 at 2 minutes and ΔpH i 0.01 ± 0.01 at 3 minutes, P<.05 versus absence of inhibitors, n=5). D, The increased early recovery during abrupt reduction in fluid shear stress was inhibited by removal of buffer Na⁺ and replacement by equimolar choline (ΔpH i 0.00 ± 0.01 at 2 minutes and ΔpH i 0.01 ± 0.02 at 3 minutes, P<.05 versus Na⁺-containing solution, n=5).

Figure 4. Effect of an abrupt decrease in flow on recovery from an NH₄Cl-induced acid load. A, Averaged data show that under conditions of continuous shear stress at 2.7 dyne/cm², there is no recovery from the acid load in the first 2 minutes (ΔpH i 0.00 ± 0.00, n=19; only data paired with an NH₄Cl-induced acid load in which flow was reduced during recovery are included). B, When fluid shear stress was abruptly reduced from 2.7 to 0.3 dyne/cm² during recovery from the acid load, pH i increased by 0.04 ± 0.02 at 2 minutes (P<.05 vs control, n=19). C, The increased early recovery during abrupt reduction in fluid shear stress was partially inhibited by the combination of DIDS and EIPA (ΔpH i 0.02 ± 0.01 at 2 minutes and ΔpH i 0.01 ± 0.01 at 3 minutes, P<.05 versus absence of inhibitors, n=5). D, The increased early recovery during abrupt reduction in fluid shear stress was inhibited by removal of buffer Na⁺ and replacement by equimolar choline (ΔpH i 0.00 ± 0.01 at 2 minutes and ΔpH i 0.01 ± 0.02 at 3 minutes, P<.05 versus Na⁺-containing solution, n=5).

The enhanced recovery during conditions of an abrupt decrease in fluid shear stress was dependent on the presence of bicarbonate in the buffer, since in bicarbonate-free solution there was no difference in the rate of recovery during continuous shear stress at 2.7 dyne/cm² (n=4) compared with conditions of an abrupt reduction in fluid shear stress from 2.7 to 0.3 dyne/cm² (n=5, P=NS, not shown). In the presence of bicarbonate, DIDS pretreatment had no effect on the rate of recovery from an NH₄Cl-induced acid load during an abrupt reduction in flow (ΔpH i 0.04 ± 0.02 at 2 minutes, P=NS versus no DIDS, n=4). Thus, a DIDS-insensitive acid extruder like the Na⁺-H⁺ exchanger may also be activated under these conditions of reduced pH i (6.95 ± 0.04 after NH₄Cl washout in DIDS), since Na⁺-H⁺ exchange activity is increased at low pH i.20 Since pH i recovery from an acid load in the presence of bicarbonate may be mediated by parallel activation of bicarbonate transporters and the Na⁺-H⁺ exchanger21,22 and since the combination of DIDS and EIPA prevented recovery from the NH₄Cl-induced acid load at a constant above normal control pH i before recovery toward baseline (Figure 5).
shear stress of 2.7 dyne/cm² (see Figure 3), the effect of this combination of inhibitors on recovery from an acid load during flow reduction was examined. As shown in Figure 4C, DIDS and EIPA inhibited the enhanced recovery when fluid shear stress was abruptly decreased from 2.7 to 0.3 dyne/cm². Shear stress was then abruptly decreased to 0.3 dyne/cm² at the point of maximal acidification after NH₄Cl washout. In 4 of 19 monolayers, the more rapid recovery resulted in an “alkaline overshoot” in which pH exceeded that during NH₄Cl exposure.

Discussion
The acute and chronic adaptation to changes in blood flow occur as a result of endothelium-dependent responses that regulate vascular tone and the organization of the blood vessel wall. Among the most rapid responses of vascular endothelial cells to changes in fluid shear stress are K⁺ channel activation, generation of inositol trisphosphate, an increase in [Ca²⁺], and changes in pH. The present study shows that an abrupt decrease in fluid shear stress produces cytosolic alkalization of vascular endothelial cells by affecting membrane ion transporters that contribute to pH regulation. After a period of exposure to continuous fluid shear stress at 2.7 dyne/cm², the abrupt reduction of fluid shear stress from 2.7 to 0.3 dyne/cm² produced a bicarbonate-dependent increase in pH of 0.27 pH unit, which was not affected by prior inhibition of the Na⁺-H⁺ exchanger alone. Recovery from an NH₄Cl prepulse–induced acid load occurred more rapidly when fluid shear stress was abruptly reduced from 2.7 to 0.3 dyne/cm² than when shear stress was continuous at 2.7 dyne/cm². This accelerated pH recovery was blocked by combined inhibition of extracellular Na⁺–dependent Cl⁻–HCO₃⁻ exchange and Na⁺-H⁺ exchange, indicating that the bicarbonate-dependent DIDS-sensitive exchange of extracellular Na⁺ and HCO₃⁻ for intracellular H⁺ and Cl⁻ (extracellular Na⁺–dependent Cl⁻–HCO₃⁻ exchange) and the amiloride-sensitive Na⁺-H⁺ exchanger are involved in the increase in endothelial pH, produced by a reduction in fluid shear stress.

The NH₄Cl prepulse method of intracellular acidification was used to assess the involvement of the extracellular Na⁺–dependent Cl⁻–HCO₃⁻ exchanger in the response to acute reduction in fluid shear stress, since DIDS pretreatment irreversibly and nonspecifically inhibits anion exchangers, including the extracellular Na⁺–independent Cl⁻–HCO₃⁻ exchanger. Thus, if monolayers had been pretreated with DIDS before the initial exposure to an increase in shear stress (as in Figure 1), the initial intracellular acidification induced by an increase in fluid shear stress would have been inhibited as well, making any effect on pH during the subsequent period of flow reduction difficult to interpret.

Recovery from an NH₄Cl prepulse–induced acid load proceeds relatively slowly in rat aortic endothelial cells exposed to continuous fluid shear stress. When rat aortic endothelial cells are cultured in microcapillary tubes and exposed to NH₄Cl and washout during constant laminar flow, no apparent pH recovery occurs in the first 2 minutes after maximal acidification. This slow early recovery contrasts with the effect observed when fluid shear stress is rapidly reduced after acid loading. Experiments in which the extracellular Na⁺–dependent Cl⁻–HCO₃⁻ exchanger and the Na⁺-H⁺ exchanger were both pharmacologically inhibited or blocked by removal of buffer Na⁺ indicate that these exchangers contribute to the accelerated pH recovery when fluid shear stress is abruptly reduced. As shown in Figure 4, DIDS and EIPA inhibited, but did not abolish, the more rapid early recovery from the acid load during flow reduction, suggesting the possibility that other acid extruders may be involved as well. One such transporter is the plasmalemmal vacuolar-type H⁺-ATPase, which regulates pH in macrophages by extruding cytoplasmic H⁺ across the plasma membrane. The selective inhibitor of the H⁺-ATPase, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (10 μmol/L), did not affect the more rapid early pH recovery when fluid shear stress was reduced during washout of an NH₄Cl prepulse either alone (n = 3, P = NS versus no inhibitor) or in combination with DIDS and EIPA (n = 3, P = NS versus DIDS and EIPA alone).
extracellular Na\(^+\)-dependent Cl\(^-\)-HCO\(_3\)\(^-\) exchanger in cerebral microvascular endothelial cells, the involvement of all three acid-base transporters (the Na\(^+\)-H\(^+\) exchanger and the extracellular Na\(^+\)-dependent and extracellular Na\(^+\)-independent Cl\(^-\)-HCO\(_3\)\(^-\) exchangers) in the regulation of pH\(_i\) in endothelial cells has not been reported, as it has in vascular smooth muscle cells.\(^{33}\) Thus, the activity of three distinct plasma membrane ion exchangers is regulated by alterations in fluid shear stress, providing a novel mechanism whereby the vascular endothelium rapidly transduces alterations in flow.

Although considerable attention has focused on the effect of increases in fluid shear stress on endothelium-dependent vasodilation\(^1\) and altered gene expression,\(^6\)--\(^9\) it is reduced flow and low fluid shear stress that are typically associated with intimal hyperplasia,\(^{12,35}\) accelerated atherosclerosis,\(^{13–15}\) and endothelium-dependent vasoconstriction.\(^11\) Our results suggest that pH\(_i\) plays an important role in endothelial mechanoreception and that the cytosolic alkalization produced by a reduction in fluid shear stress may function as an intracellular signaling mechanism linking flow reductions to changes in vascular tone and intimal thickening. Endothelium-dependent vasodilatation to acetylcholine is inhibited by intracellular alkalization.\(^{36}\) Expression of PDGF, which is both a potent smooth muscle cell mitogen and a vasoconstrictor,\(^37\) is induced by reduced shear stress in vivo,\(^38\) and the proliferative response in neointimal smooth muscle cells initiated by a reduction in fluid shear stress may be stimulated by endothelial production of PDGF.\(^39\) Endothelial production of PDGF might be facilitated by the intracellular alkalization that occurs during an abrupt reduction in fluid shear stress, since thrombin-stimulated PDGF production has been shown to be activated by endothelial Na\(^+-\)H\(^+\) exchange activity, which would be expected to increase endothelial pH\(_i\).\(^{39}\) Further studies are needed to address the functional significance of the endothelial pH\(_i\), changes produced by reductions in fluid shear stress.

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