Cellular Responses to Mechanical Stress
Invited Review: Effects of flow on vascular endothelial intracellular calcium signaling of rat aortas ex vivo

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Jen, Chauying J., Shuo-Ju Jhiang, and Hsiun-Ing Chen. Effects of flow on vascular endothelial intracellular calcium signaling of rat aortas ex vivo. J Appl Physiol 89: 1657–1662, 2000.—To study the effects of flow on in situ endothelial intracellular calcium concentration ([Ca$^{2+}$]$_i$) signaling, rat aortic rings were loaded with fura 2, mounted on a tissue flow chamber, and divided into control and flow-pretreated groups. The latter was perfused with buffer at a shear stress of 50 dynes/cm$^2$ for 1 h. Endothelial [Ca$^{2+}$]$_i$ responses to ACh or shear stresses were determined by ratio image analysis. Moreover, ACh-induced [Ca$^{2+}$]$_i$ elevation responses were measured in a calcium-free buffer, or in the presence of SKF-96365, to elucidate the role of calcium influx in the flow effects. Our results showed that 1) ACh increased endothelial [Ca$^{2+}$]$_i$ in a dose-dependent manner, and these responses were incremented by flow-pretreatment; 2) the differences in ACh-induced [Ca$^{2+}$]$_i$ elevation between control and flow-pretreated groups were abolished by SKF-96365 or by Ca$^{2+}$-free buffer; and 3) in the presence of 10$^{-5}$ M ATP, shear stress induced dose-dependent [Ca$^{2+}$]$_i$ elevation responses that were not altered by flow-pretreatment. In conclusion, flow-pretreatment augments the ACh-induced endothelial calcium influx in rat aortas ex vivo.

flow pretreatment; calcium image; acetylcholine; shear stress; endothelial cells

ENDOTHELIAL CELLS CAN PRODUCE at least three kinds of endothelium-derived relaxing factors (EDRF) to modulate vascular tone, i.e., nitric oxide (NO), endothelium-derived hyperpolarization factor, and prostacyclin (37). Numerous factors, including shear stress (or flow) and receptor-mediated agonists such as ACh, are capable of stimulating EDRF release and/or causing vasodilation (11, 12, 17, 26). Although shear stress has been known to affect many endothelial parameters, such as K$^+$ channel opening (27) and intracellular pH (39), this study focuses on its effect on the intracellular calcium concentration ([Ca$^{2+}$]$_i$) elevation. Recent animal studies have reported that flow or ACh increased endothelial [Ca$^{2+}$]$_i$ levels and dilated isolated arterioles in rats or in rabbits (9, 25). Studies on cultured endothelial cells or on an isolated arteriole of the rat have shown that fluid shear stress causes elevation of endothelial [Ca$^{2+}$]$_i$ (8, 31, 33). Previous studies using cultured human umbilical vein endothelial cells also indicated that histamine-stimulated EDRF release requires calcium influx (18, 19). In addition, receptor-regulated endothelial NO synthase (NOS) translocation and activation need endothelial [Ca$^{2+}$]$_i$ elevation as well (29). Therefore, the endothelial [Ca$^{2+}$]$_i$ signaling should play an important role in these endothelial functions.

Previous studies have indicated that exercise enhances agonist-stimulated, endothelium-dependent vasorelaxation responses (2–4, 7). Interestingly, these exercise effects have been observed in aortas and pulmonary arteries but not in carotid arteries (3). Regional increases in blood flow during exercise may thus play an important role. As mentioned previously, calcium signaling is important in mediating endothelium-dependent vasodilating responses. Whether exercise effects are mediated by changes in agonist-evoked endothelial [Ca$^{2+}$]$_i$ responses cannot be easily ascertained using cultured endothelial cells, because these cells fail to respond to muscarinic agonist administration with either an increase in [Ca$^{2+}$]$_i$, or a release of EDRF (20, 28). Besides, cultured endothelial cells express different muscarinic receptor mRNAs (35). Recently, our laboratory developed an in situ calcium imaging method, with single-cell resolution, to examine the endothelial [Ca$^{2+}$]$_i$ signaling in rat aortas (14).
Moreover, a previous study from our laboratory showed that exercise increased ACh-induced endothelial \([Ca^{2+}]_{i}\) responses by facilitating calcium influx (5). Therefore, the present study is designed to investigate the effect of flow-pretreatment, which simulates the flow condition in exercise, on endothelial cell \([Ca^{2+}]_{i}\) responses to ACh.

**MATERIALS AND METHODS**

**Animals and vessel preparation.** This study was conducted in conformity with the *Guiding Principles in the Care and Use of Animals*. Six- to eight-week-old male Sprague-Dawley rats were purchased from National Cheng-Kung University Animal Center (Tainan, Taiwan). The rats were anesthetized with ether anesthesia and killed by decapitation. The thoracic aorta was then isolated and cut into 5-mm-long vessel rings. After removal, the aorta was placed in an organ chamber containing Krebs-Ringer solution bubbled with 95% O\(_2\)-5% CO\(_2\) (22°C, pH 7.4). This solution had the following composition (in mM): 118.0 NaCl, 4.8 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 24 NaHCO\(_3\), 0.03 Na\(_2\)EDTA, and 11.0 glucose.

Aortic rings were fluorescently labeled by incubating with 10 \(\mu\)M of fura 2 AM and 0.025% pluronic F-127 in Krebs-Ringer solution for 1 h (36). Extracellular fura 2 AM was washed out afterward. After fura 2 loading, vessel rings were divided into control and flow-pretreated groups, then cut open and pinned to the baseplate of a flow chamber (5, 14). We made the dent on the cover plate deeper to accommodate the extra thickness of tissue. Although this gap, strictly speaking, was not fixed, it was estimated to be within 10% of 0.15 mm. After tissue mounting, the true gap thickness was monitored under a microscope, and the flow rate was adjusted accordingly to be 50 dyn/cm\(^2\) for each flow-pretreated specimen.

The flow-pretreated vessel segments were perfused with Krebs-Ringer solution at a shear stress of 50 dyn/cm\(^2\) for 1 h, whereas the controls were not pretreated with any flow before the experiments detailed below.

**Measurement of in situ endothelial \([Ca^{2+}]_{i}\).** The setup for endothelial \([Ca^{2+}]_{i}\) image analysis described in previous studies by our laboratory was used (5, 14). The flow chamber, mounted with either control or flow-pretreated vessel segment, was placed on an inverted microscope with epifluorescence attachments (Diaphot 300, Nikon, Tokyo, Japan). The excitation light from a xenon lamp was filtered with a high-speed rotating filter wheel (Lambda 10–2, Sutter, Novato, CA) to provide wavelengths of 340 and 380 nm. The fluorescence images at 510 nm were recorded by a high-sensitivity speed rotating filter wheel (Lambda 10–2, Sutter, Novato, CA) to provide wavelengths of 340 and 380 nm. The fluorescence at 380 nm with calcium-free solution to that of saturated CaCl\(_2\) solution. All experiments were conducted at room temperature.

\([Ca^{2+}]_{i}\) elevation responses to ACh. After the vascular endothelial cells had been focused properly, fresh Krebs-Ringer buffer was perfused through the chamber at a flow rate of 0.05 ml/min. At the same flow rate, dose responses of ACh-induced \([Ca^{2+}]_{i}\), elevation were determined by subsequent applications of ACh (from \(10^{-10}\) to \(10^{-6}\) M). Between each ACh application, the chamber was washed with fresh buffer for \(~4\) min to recover the basal \([Ca^{2+}]_{i}\), level. The results between control and flow-pretreated groups were compared by off-line image analysis.

**Role of calcium influx in ACh-evoked \([Ca^{2+}]_{i}\), response.** The ACh (\(10^{-8}\) M)-evoked \([Ca^{2+}]_{i}\), response was evaluated in the presence or absence of 30 \(\mu\)M of SKF-96365, a membrane calcium channel blocker. Calcium-free buffer containing 0.1 mM EGTA was used as the perfusion buffer in some experiments to evaluate the role of calcium influx in ACh-induced \([Ca^{2+}]_{i}\), response. The ACh (\(10^{-8}\) M)-evoked \([Ca^{2+}]_{i}\), responses to shear stress were analyzed by repeated-measures ANOVA. Differences between control and flow-pretreated groups were compared by using an unpaired Student’s t-test, with \(P < 0.05\) considered to be statistically significant.

**RESULTS**

**Endothelial \([Ca^{2+}]_{i}\), responses to ACh.** ACh reversibly induced endothelial \([Ca^{2+}]_{i}\), elevation in the rat aortic endothelium, and this elevation increased with in-
creasing ACh concentrations (Fig. 1). It was noticed that flow-pretreatment of dissected vessel segments enhanced their endothelial \([Ca^{2+}]_i\) responses to ACh. The average values of dose responses of ACh-stimulated \([Ca^{2+}]_i\) elevation in control and flow-pretreated groups are shown in Fig. 2.

The basal endothelial \([Ca^{2+}]_i\) level was quite homogeneous, between 50 and 150 nM in most cells. When the histograms of \([Ca^{2+}]_i\) responses in 100 individual endothelial cells from one set of experiments were analyzed, it was clear that individual endothelial cells on the same endothelium had heterogeneous responses to ACh (Fig. 3). However, whereas certain cells responded to ACh application with a several-fold increase in \([Ca^{2+}]_i\) level, some cells were relatively unresponsive to ACh. The higher the concentration of ACh, the more widespread the cell number was distributed. This histogram shift was more pronounced in the flow-pretreated group than the control; i.e., more cells had greater ACh-stimulated \([Ca^{2+}]_i\) elevation responses than the controls.

**Role of calcium influx in ACh-evoked \([Ca^{2+}]_i\) responses.** SKF-96365, a calcium influx blocker, alone did not influence the basal endothelial \([Ca^{2+}]_i\) (without SKF-96365: 125 ± 14 nM control, 128 ± 25 nM flow-pretreated; with SKF-96365: 130 ± 11 nM control; 123 ± 15 nM flow-pretreated, \(n = 5\) for each group). ACh-evoked \([Ca^{2+}]_i\) responses in the presence or absence of SKF-96365 were compared between control and flow-pretreated groups. Table 1 demonstrates that flow-pretreatment increased ACh-evoked endothelial \([Ca^{2+}]_i\) responses and that this effect disappeared after administration of SKF-96365. If a calcium-free buffer substituted the Krebs-Ringer solution, minimal ACh-induced calcium elevation was observed in both groups (Table 1). Therefore, the enhancement of ACh-evoked endothelial \([Ca^{2+}]_i\) responses by flow-pretreatment was mainly due to an increase in calcium influx.

**[Ca^{2+}]_i** elevation responses to shear stress in the presence of ATP. Figure 4 demonstrates that, in the presence of \(10^{-5}\) M ATP, different levels of shear stress directly evoked different extents of endothelial cell \([Ca^{2+}]_i\) elevation. The average “dose-response” curves

![Fig. 1. Examples of ACh-induced intracellular calcium concentration ([Ca^{2+}]_i) elevation response tracings in control (A) and flow-pretreated (B) groups. Signals were obtained from areas covering ~250 endothelial cells in the mainstream region of rat thoracic aortas.](image)

![Fig. 2. Comparison of dose-response relations of ACh-induced [Ca^{2+}]_i elevation responses between control (○; \(n = 10\)) and flow-pretreated (●; \(n = 10\)) groups. Results were analyzed by repeated-measures ANOVA (\#\(P < 0.05\)), followed by unpaired Student’s t-test at given doses of ACh (*\(P < 0.05\)).](image)

![Fig. 3. Histograms of basal or ACh-induced [Ca^{2+}]_i levels in 100 endothelial cells from 1 control (A) and 1 flow-pretreated (B) specimen.](image)
of control and flow-pretreated groups are shown in Fig. 5. There was no significant difference between these two groups.

**DISCUSSION**

Previous reports of endothelial cell calcium signaling in response to agonists or to flow were mainly based on studies using cultured cells (16, 18, 19, 24, 31, 33). However, cultured endothelial cells may lose their inherent properties and behave differently from the vascular endothelium (20, 28, 35). We therefore developed an in situ calcium imaging method (with single-cell resolution) to study endothelial cell calcium signaling. By analyzing calcium images of rat thoracic aortic vascular endothelium ex vivo, we are the first to report that 1) flow pretreatment enhances ACh-induced \([\text{Ca}^{2+}]_i\) elevation; 2) the differences between control and flow-pretreated groups in ACh-evoked \([\text{Ca}^{2+}]_i\) responses were abolished by SKF-96365 or by \([\text{Ca}^{2+}]_i\)-free buffer; 3) in the presence of ATP, shear stress also

![Fig. 4. Examples of shear stress-induced \([\text{Ca}^{2+}]_i\) elevation response tracings in the presence of ATP (10^(-5) M) for control (A) and flow-pretreated (B) groups.](Image)

![Fig. 5. Comparison of dose-response relations of shear stress-induced \([\text{Ca}^{2+}]_i\) elevation responses between control (○, n = 5) and flow-pretreated (●, n = 6) groups.](Image)
pre-treatment are unclear at the present time. However, one can speculate that the modulation of either calcium channel activity or ACh receptors (i.e., M₃ receptors) may account for this effect. If the calcium channel activity itself was altered by flow pre-treatment, one would expect to see an increase in shear-induced endothelial calcium responses as well. However, because our flow and ATP results (Figs. 4, 5) are against this viewpoint, it is likely that M₃ receptors are upregulated instead. The previous study by our laboratory showed that acute exercise enhances ACh-evoked endothelium-dependent vasorelaxation by M₃ receptor upregulation in rat aortas (4). Whether flow-pre-treatment also upregulates endothelial M₃ receptors is still unknown. In 1997, Takada et al. (34) reported that cultured human umbilical vein endothelial cells exposed to fluid shear stress increased gene expression of the G protein-coupled receptors EDG1 and FEG1. In their study, the level of EDG1 mRNA began to increase as early as 1 h after exposure to shear stress. Because the muscarinic receptor is one of the G protein-coupled receptors (38), we favor the possibility of upregulating muscarinic receptors by flow-pre-treatment.

In this study, we also confirmed that ACh evoked endothelial [Ca²⁺]ᵢ elevation in a dose-dependent manner, which is consistent with previous animal studies (9, 25). Nonetheless, Falcone et al. (9) and Muller et al. (25) reported that shear stress alone (in the absence of exogenous ATP) increased endothelial [Ca²⁺]ᵢ in rat cremaster arterioles or in rabbit coronary arterioles. In the present study, shear, by itself, did not induce endothelial [Ca²⁺]ᵢ elevation in rat aorta, which is consistent with a previous report that also used the rat aorta (21). It appears that the direct shear effect only occurs in small arterioles, not in large vessels. The concept of a regional difference in endothelial [Ca²⁺]ᵢ signaling is supported by our laboratory’s recent report, in which endothelial heterogeneity between branch and nonbranch regions was observed (14). Besides, when cultured endothelial cells from large vessels are used, shear evokes significant endothelial [Ca²⁺]ᵢ elevation only if the perfusion solution contains ATP (16, 24).

Complicated forms of [Ca²⁺]ᵢ signaling, such as single transients and repeated spikes with variable frequencies, have been reported in studies that mainly used cultured cells (1). However, unlike a previous study on cultured single bovine aortic endothelial cells (33), we rarely observed [Ca²⁺]ᵢ oscillations in single vascular endothelial cells in situ, either under shear (present study) or with agonist application (14). Therefore, the physiological significance of endothelial [Ca²⁺]ᵢ oscillation remains to be elucidated. A previous study indicated that administration of atropine or acetylcholinesterase would inhibit flow-induced NO release and vasorelaxation (22). The results of that study imply that besides the flow-mediated mechanotransduction (6), local ACh release from the endothelial cells may also mediate the flow effect. It would be interesting to examine the role of endothelial [Ca²⁺]ᵢ in this aspect.

Despite the fact that flow-pre-treatment did not alter direct effects of the shear stress-induced endothelial [Ca²⁺]ᵢ elevation, we cannot rule out the possibility that flow-pre-treatment increases NO release by activating NOS in a Ca²⁺-independent manner or enhancing NOS gene expression. Recent studies have shown that fluid shear stress can activate NOS either by rapid caveolin dissociation and calmodulin association (30), or in a Ca²⁺-independent, but tyrosine phosphatase inhibitor-sensitive, manner (10). Besides, exposure to flow for a period of time can upregulate endothelial NOS mRNA in cultured endothelial cells (26).

In conclusion, flow-pre-treatment, used to simulate the flow condition in exercise, enhances the ACh-induced endothelial [Ca²⁺]ᵢ elevation in situ by causing a greater calcium influx. On the contrary, flow-pre-treatment does not change shear stress-induced endothelial [Ca²⁺]ᵢ responses.

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