Fluid shear stress increases membrane fluidity in endothelial cells: a study with DCVJ fluorescence

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Haidekker, Mark A., Nicolas L’Heureux, and John A. Frangos. Fluid shear stress increases membrane fluidity in endothelial cells: a study with DCVJ fluorescence. Am J Physiol Heart Circ Physiol 278: H1401–H1406, 2000.—Fluid shear stress (FSS) has been shown to be an ubiquitous stimulator of mammalian cell metabolism. Although many of the intracellular signal transduction pathways have been characterized, the primary mechanoreceptor for FSS remains unknown. One hypothesis is that the cytoplasmic membrane acts as the receptor for FSS, leading to increased membrane fluidity, which in turn leads to the activation of heterotrimeric G proteins (13). 9-(Dicyanovinyl)-julolidine (DCVJ) is a fluorescent probe that integrates into the cell membrane and changes its quantum yield with the viscosity of the environment. In a parallel-plate flow chamber, confluent layers of DCVJ-labeled human endothelial cells were exposed to different levels of FSS. With increased FSS, a reduced fluorescence intensity was observed, indicating an increase of membrane fluidity. Step changes of FSS caused an approximately linear drop of fluorescence within 5 s, showing fast and almost full recovery after shear cessation. A linear dose-response relationship between shear stress and membrane fluidity changes was observed. The average fluidity increase over the entire cell monolayer was 22% at 26 dyn/cm². This study provides evidence for a link between FSS and membrane fluidity changes and suggests that the membrane is an important flow mechanosensor of the cell.

parallel-plate flow chamber; molecular rotors; fluorescent probes; mechanoreceptor

ENDOTHELIAL CELLS are continuously exposed to fluid flow and the associated mechanical forces (26). They are able to sense fluid flow and effect changes that serve to maintain a specific level of flow or associated shear stress in blood vessels (8). Transfer of fluid flow forces to the cell occurs at the luminal cell surface, with the plasma membrane and associated transmembrane proteins likely candidates for mechanoreceptors that transduce the extracellular stimulus into intracellular biochemical signals.

Fluid flow stimulates numerous responses in endothelial cells. These include elevated production of the second messengers inositol 1,4,5-trisphosphate (3, 27, 29) and cyclic guanosine monophosphate (18); increased release of vasoactive compounds like prostacyclin (PGI₂) (10), nitric oxide (19), and endothelin-1 (17); increased MAP kinase activity (1, 30); elevated levels of platelet-derived growth factor (14); and c-fos gene expression (15). Many of these flow-induced responses are mediated by G protein activation, as demonstrated by their inhibition by guanosine 5’-O-(2-thiodiphosphate) (4, 15, 18), a non-hydrolyzable analog of GDP. Pertussis toxin (PTX) inhibited flow-induced production of PGI₂ (4) and endothelial nitric oxide synthase gene expression (24), while having no effect on nitric oxide or cGMP production (19). This indicates that both PTX-sensitive and -insensitive G proteins are activated by flow.

Although intracellular events triggered by fluid shear have been elucidated, the primary mechanosensor which transduces this mechanical stimulus into a biochemical signal remains unknown. Many mechanisms have been proposed (see Ref. 8 for review). This includes the direct stimulation of transmembrane proteins exposed on the luminal surface or distorted within the strained membrane (including G protein receptors), activation of ion channels which alter membrane polarization or intracellular calcium, transduction of stress along cytoskeletal elements to other regions of the cell (focal adhesions, nuclear membrane), and changes in the physical properties of the plasma membrane itself under flow. The activation of ion channels may be caused by an increase of membrane fluidity (2, 6). This is also consistent with the finding that shear stress activates potassium ion channels in endothelial cells (28). While indirect evidence or secondary events have been used to infer their involvement, no direct evidence of the listed mechanisms at the beginning of the signaling pathways due to fluid shear has been demonstrated. Previously, it was found that shear stress caused increased membrane permeability to the amphipatic dye merocyanine 540,
suggesting a flow-induced increase in membrane fluidity (5). However, differences in the membrane permeability were mainly found after 5 min of shear exposure. Another recent study (13) has shown that shear stress activates heterotrimeric G proteins reconstituted in liposomes, in the absence of any other receptors, proteins, or cytoskeletal components. Furthermore, it was shown that the activation of G protein could also be accomplished by agents which increase membrane fluidity (13). That study demonstrates the most elementary components necessary for mechanochemical signal transduction: a phospholipid bilayer and heterotrimeric G proteins.

9-(Dicyanovinyl)-julolidine (DCVJ) is a fluorescent molecular rotor with a quantum yield \( \Phi \) that is related to the viscosity \( \eta \) of the environment following the Förster-Hoffmann-equation, Eq. 1 (9)

\[
\log \Phi = C + x \log \eta
\]

where \( x \) is a molecule-dependent constant, and \( C \) is a term reflecting the relaxation rates and the temperature (22). Experiments that were conducted in media with different viscosities have confirmed the above equation (20) and also showed that the change of quantum yield with viscosity is significant. The quantum yield increases approximately by a factor of 30 when dissolved in 1-propanol and glycerol, respectively (20). The change of quantum yield occurs within a nanosecond time span (23), and can thus be considered instantaneous. This property suggests that DCVJ is a well-suited probe to assess the viscosity of phospholipid bilayers (20, 23). In this study, DCVJ was used to monitor changes in membrane fluidity that are caused by fluid shear stress (FSS).

**MATERIALS AND METHODS**

Reagents and cell media were purchased as follows: DCVJ from Molecular Probes (Eugene, OR), Hanks’ buffered saline solution (HBSS) without phenol red and M199 from Gibco (Grand Island, NY), nonenzymatic cell-dissociation solution, spectroscopic grade methanol and dextran (molecular weight 2,000,000) from Sigma (St. Louis, MO), and FCS from BioCell (Rancho Dominguez, CA).

For the determination of the relationship between quantum yield and viscosity of the medium, mixtures of ethylene glycol and glycerol were prepared. Different viscosities were achieved through different mixture ratios of ethylene glycol- to-glycerol (vol/vol) as follows: 7:3 (49 cP), 5:5 (115 cP), 4:6 (163 cP), 3:7 (245 cP), and 2:8 (391 cP), according to an experiment described previously (16, 21). Each of these mixtures (3.5 ml of each) was filled into a spectroscopic cuvette under addition of 10 µl of DCVJ stock solution, which is 10 mM DCVJ in methanol. The emission intensity of each of the five samples was acquired using a RF-1501 Fluorophotometer (Shimadzu, Kyoto, Japan) set at an excitation wavelength of 455 nm and an emission wavelength of 505 nm.

Human umbilical vein endothelial cells (HUVECs) were harvested and grown to confluency (5). Once confluency was achieved, the cells were lifted with cell dissociation solution and resuspended in medium 199 (M199) with 20% FCS at a concentration of \( 2.5 \times 10^6 \) cells/ml. Aliquots of 350 µl of the suspension were used to seed the cells on glass slides (dimensions 10 × 40 mm) that were previously treated for 2 h with 0.5 M NaOH. The cells attach and form a confluent layer within 24 h.

A staining solution was prepared from 100 µl stock solution of 10 nM DCVJ in methanol, which was dispersed in 2 ml FCS under vigorous stirring. M199 (10 ml) was added. The slides with cells were covered with the staining solution. Incubation took place over 2 h in the dark at 37°C with the fluid in slow constant circular motion. After the incubation period, the cells were rinsed with HBSS, and the fluorescence checked under an epifluorescent microscope (Diaphot TMD, Nikon, Garden City, NY) using the G2B filter set. Flow chambers were assembled using a standard methacrylate spectroscopic cuvette (Fisher Scientific, Pittsburgh, PA) and a parallel-plate black Delrin flow channel with a channel width of 6 mm and a depth of 500 µm. Excitation wavelength was set to 455 nm and emission wavelength to 505 nm. An additional 475 nm LP emission filter (Chroma, Brattleboro, VT) blocked scattered blue light. A microcontroller-driven syringe pump (Pump 22, Harvard Apparatus, Holliston, MS) attached to a PC provided a controlled flow of the flow medium, HBSS. Before entering the flow chamber, the flow medium was preheated to 37°C in a controlled-temperature heat exchanger. Throughout the experiments, a minimum flow of 1 ml/min was maintained to provide the cells with fresh medium. This minimal flow generated a shear stress of 0.7 dyn/cm². Photobleaching of the probe caused a long-term decay of the fluorescence intensity at a rate of up to 1% per min. This decay has been mathematically corrected using Eq. 2 (see also Figs. 3 and 4)

\[
I_c(t) = I_c(0) e^{-t/t_c} \tag{2}
\]

where \( I_c(t) \) is the raw intensity time course, \( I_c(0) \) the compensated time course, and \( t_c \) is the compensation time constant with a value of 100 min. This value was determined by recording the fluorescence intensity change for 5 min before the actual measurement and fitting an exponential curve to the data.

In addition to experiments with different flow rates, a flow medium that consisted of 2.5 wt% of dextran in HBSS was used to modulate viscosity and thus the shear stress without changing the flow rate. The viscosity of the dextran medium was determined to be 3.8-fold higher than dextran-free HBSS (falling ball viscometer, size 1, Gilmot Instruments, Barrington, IL). The following sequence was used to apply different shear stresses to the cell monolayer: 1) initial flow of 2 ml/min dextran-free medium (1.3 dyn/cm²), 2) 20 ml/min (12.7 dyn/cm²) dextran-free medium, 3) 14 ml/min medium with 2.5% dextran and 6 ml/min medium without dextran (35 dyn/cm² with 20 ml/min total flow), and 4) 20 ml/min dextran-free medium (12.7 dyn/cm²). The viscosity of the mixture was determined to be 2.7-fold higher than dextran-free HBSS. Two identical syringe pumps were configured as a pump chain and controlled by the same computer to achieve the flow profiles. The perfusion fluids were fed into a mixer before reaching the temperature controller unit. The properties of the mixing process were observed using ink-marked fluids.

Other experiments were performed to determine the effect of agents that alter membrane fluidity (13). Cells were stained for 1 h at 37°C in the dark while suspended in the above staining solution. After the staining period, the staining solution was removed by centrifuging the cells for 10 min at 1,100 rpm and resuspending them in M199. Membrane fluidity of the suspended cells was altered by progressively adding benzyl alcohol (13) to a cell suspension in a spectroscopic cuvette. The suspension was stirred gently throughout
the experiment except during the actual data acquisition. Intensity values were acquired 3 min after each addition of benzyl alcohol.

The statistical analysis was performed using the software package Prism 3.0 (Graphpad, San Diego, CA). This included the least-squares linear fit, the Kolmogorov-Smirnov test for Gaussian distribution of the data, and the t-test to test whether responses to shear stress were significantly different from zero.

RESULTS

DCVJ exhibits a dependency of its emission intensity on the viscosity of the solvent. This dependency is described by Eq. 1. With the use of media with different viscosities, it was possible to verify the double logarithmic relationship of quantum yield and viscosity as can be seen in Fig. 1. The slope of the line was determined as 0.58 (r² = 0.98), which is in good accordance with the value of 0.6 found by Förster and Hoffmann (9).

The cells stained in suspension showed a strong fluorescence under the microscope and in the fluorophotometer. Repeated addition of benzyl alcohol caused a decrease in fluorescence intensity, indicating an increase of the fluidity of the DCVJ environment, the cell membrane (Fig. 2, inset).

In the flow chamber, different step-shaped flow profiles were applied to the cell layer that was grown confluent on the flow-chamber glass plates. In all cases, a marked decrease of emission intensity could be observed when flow was increased, followed by a recovery of the intensity when the flow was decreased. The intensity drop and recovery were almost linear, leveling off ~5 s after the onset of flow (Fig. 3). In unstained control cells, this change could not be observed.

In the examined shear stress range (0.7 to 33 dyn/cm²), an increase in shear stress leads to a corresponding increase in membrane fluidity (Fig. 3), as shown through decreased DCVJ fluorescence intensity. For each phase of constant flow, a time-averaged intensity was calculated at steady state (after the first 5 s). For each positive transition of flow, a difference between the high-intensity level (0.7 dyn/cm²) and the lower intensity level caused by shear stress was calculated, resulting in data points of intensity drop over shear stress. To eliminate random influences of the different experiments (background, cell density, staining intensity), the data points were normalized by the intensity drop measured when FSS changes from 0.7 to 26 dyn/cm². These intensity drops were averaged over nine similar experiments (Fig. 2). The data points lie approximately on a straight line (slope 0.038 ± 0.003, different from zero with P < 0.001), which can be obtained by linear regression. The regression coefficient was calculated as r² = 0.96.

For an independent confirmation that shear stress causes higher membrane fluidity, media with different viscosities (HBSS and HBSS containing dextran) were used to change the shear stress while maintaining a constant flow rate. Figure 4 shows the fluorescence intensity with the application of shear stress in a typical experiment. The intensity drop at step 3 (fluid containing dextran) is 3.7-fold stronger than the drop.
at step 2, although the flow rate is kept constant at 20 ml/min. This intensity drop is of the same order of magnitude (4.3) as the one found in Fig. 2 between 12.7 and 32.7 dyn/cm².

DISCUSSION

The fluorescent dye DCVJ, a molecular rotor, exhibits a quantum yield that depends on the restriction of the rotational movement of its polar head (20). With unrestricted rotational movement, the majority of the energy of the excited state is lost nonradiatively. Increased viscosity of the environment imposes a restriction on the rotational movement. The quantum yield increases, which can be observed through an increased fluorescence intensity. This relationship has been quantitatively described by Förster and Hoffmann (9). Experiments with solvents of different viscosity confirm the relationship (16, 20). DCVJ has also been used to monitor microviscosity of micelles and DPPC vesicles (20).

In our study, a confluent layer of endothelial cells, stained with DCVJ, exhibited a decrease in fluorescence intensity when exposed to FSS. In response to a step change of flow, cells showed a 4- to 5-s adjustment phase, through which the intensity drops in an approximately linear fashion, followed by a steady state where intensity remains almost constant. It is difficult to interpret whether the quantitative relationship of the intensity drop with the shear stress is linear or not. The data points lie on a parabola (0.012x + 0.00096x²), but there is no reason to assume specific nonlinear functions. Taking into account the small coefficient of the above second-order term, and the high-correlation coefficient for the linear curve-fit (r² = 0.96), a first-order approximation was considered sufficient.

DCVJ has been shown to bind to tubulin (21), a process accompanied by an increase in quantum yield. The polymerization of tubulin further increases DCVJ quantum yield. However, the remodeling of microtubules has a longer time constant by orders of magnitude: remodeling times have been found to be between 3 and 48 h (7, 11). Because the reaction times in our experiments were found to be in the order of 5 s, and fluorescence microscopy indicated that DCVJ binding...
to the cytoskeleton was insignificant, we can exclude the influence of tubulin binding effects.

Another possible mediator of the changes in DCVJ fluorescence is shear-induced alteration of cytoplasmic viscosity, because small amounts of DCVJ migrate into the cell cytoplasm. With an experimental device different from this one, others have shown that cytoplasmic viscosity increases with mechanical probing of cells (25). Therefore, if DCVJ was dissolved in the cytoplasm, it would contribute towards the fluorescence intensity in the opposite way, increasing intensity with shear stress. This suggests that the contribution of the probe dissolved in the cytoplasm, or the viscosity change of the cytoplasm, is negligible.

A rise of temperature reduces the quantum yield (22, 23). To exclude temperature artifacts, we used a temperature controller in conjunction with a heat exchanger and a controlled-temperature heated cuvette holder. In addition to the experiments conducted at 37°C, all experiments were repeated at room temperature with the flow medium fully equilibrated to the ambient temperature. The decrease in DCVJ fluorescence intensity with increasing shear stress as presented above was observed at room temperature as well (data not shown).

For an independent confirmation that the observed intensity changes are caused by FSS and are not a transport-related phenomenon, shear stress was modulated through different media viscosities. As with increased fluid flow, an increase of shear stress caused by increased perfusion fluid viscosity resulted in a reduced fluorescence emission intensity. The proportion relationship between shear stress and intensity decrease could also be confirmed. This can also be seen in Fig. 4, where the high increase of shear stress (35 dyn/cm²) causes a far more pronounced intensity drop than the relatively lower shear stress (13 dyn/cm²) caused by the increase of flow.

The Förster-Hoffmann equation (Eq. 1) allows us to draw certain conclusions on the viscosity changes.

Assuming that the intensity measured is the addition of background intensity I₀ (scattered light and filter bleed-through) and the fluidity-related intensity I₁ (without shear) and I₂ (with shear), and that the quantum yield is reflected by the fluidity-related intensity alone (i.e., Φ₁₂ ≈ Φ₁), with an invariant proportionality constant, we use Eq. 1 to calculate the ratio of the two viscosities in Eq. 3

\[ \frac{\eta₁}{\eta₂} = \frac{\Phi₁}{\Phi₂} = \frac{I₁ - I₀}{I₂ - I₀} \]

with the constant x = 0.6 for DCVJ (9). The intensity of the reflected light was determined by removing the cells from the slide after the experiment. The intensity contribution from the cell cytoplasm could not be determined exactly and was therefore not taken into consideration. With this, the ratio of η₁ to η₂ was calculated as 0.78; the change of viscosity is therefore 22% for the shear stress of 26 dyn/cm². This value is an average over the entire cell monolayer including all regions of the cells. It is likely that there are local regions of higher fluidity (for example microdomains to the upstream cell-cell junction). To elucidate local membrane fluidity changes on a sub-cellular level, further spatial and temporal measurements employing fluorescence microscopy are required.

This study demonstrates that FSS increases membrane fluidity in the endothelial cell membrane. We hypothesize that the increase in membrane fluidity can increase the intramolecular dynamics and diffusivity of membrane-bound enzymes, such as G proteins (12, 13), which in turn results in mechanochemical transduction across the cell membrane.

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