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Fine control of endothelial VEGFR-2 activation: caveolae as fluid shear stress shelters for membrane receptors

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Abstract
Recent experimental evidence points to the possibility that cell surface-associated caveolae may participate in mechanotransduction. The particular shape of caveolae suggests that these structures serve to prevent exposure of putative mechanosensors residing within these membrane invaginations to shear stresses at magnitudes associated with initiation of cell signaling. Accordingly, we numerically analyzed the fluid flow in and around caveolae using the equation of motion for flow of plasma at low Reynolds numbers and assuming no slip-condition on the membrane. The plasma velocity inside a typical caveola and the shear stress acting on its membrane are markedly reduced compared to the outside membrane. Computation of the diffusion field in the vicinity of a caveola under flow, however, revealed a rapid equilibration of agonist concentration in the fluid inside a caveola with the outside plasma. Western blots and immunocytochemistry support the role of caveolae as shear stress shelters for putative membrane-bound mechanoreceptors such as flk-1. Our results, therefore, suggest that caveolae serve to reduce the fluid shear stress acting on receptors in their interior, while allowing rapid diffusion of ligands into the interior. This mechanism may permit differential control of flow and ligand activation of flk-1 receptor in the presence of ligands.

Keywords Caveolin · Vascular endothelial growth factor receptor · Membrane mechanics · Flow analysis · Diffusion analysis · Finite element analysis · Shear stress

1 Introduction

Caveolae are 50- to 100-nm invaginations of the plasma membrane of many cell types (Feron et al. 1996; Chigorno et al. 2000; Chang et al. 1994; Cohen et al. 2004a). Originally described with electron microscopy (Yamada 1955; Bruns and Palade 1968), some investigators proposed that caveolae are part of a membrane system that enables vesicular and/or channel-like transport for large molecules, e.g., albumin, in endothelial cells (Palade and Bruns 1968; Simionescu et al. 2002). Others suggest that caveolae could be part of a potocytosis system designed to transport macromolecules between the membrane and selected intracellular organelles (Mineo and Anderson 2001; Anderson et al. 1992). Discovery of the caveolin family of membrane proteins involved in regulating caveolar structure, together with the enriched population of surface receptors inside caveolae (Shaul and Anderson 1998; Schnitzer 2001; Lasley and Smart 2001; Feron et al. 1999), raises the possibility that these membrane invaginations serve as signaling microdomains (Lisanti et al. 1994) and facilitate such diverse functions as lipid metabolism (Cohen et al. 2004b), receptor stability (Capozza et al. 2005) and organogenesis (Park et al. 2002). Finally, there is evidence to suggest that caveolae are *mechanotransduction* or *sensing* centers for cells (Rizzo et al. 1998a, 2003; Boyd et al. 2003).

Structurally, caveolae can be readily unfolded by tension in the plasma membrane (Lee and Schmid-Schönbein 1995), and the majority of plasmalemmal "vesicles" inside the cell cytoplasm that appear to be closed (e.g., on ultrathin electron microscopic images) are actually attached to the plasmalemmal membrane and form part of the caveolar system (Bundgaard et al. 1983; Frokjaer-Jensen 1991; Chien

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et al. 1981). In spite of electron microscopic evidence for transcytosis, there is little direct evidence for movement of these structures across unstimulated cells (Thomsen et al. 2002). Some caveolae that bind low-density lipoproteins have the ability to accumulate in lysosomes in the perinuclear region (Handley et al. 1981) and thus are able to be transported inside the cell cytoplasm of endothelial cells. The available evidence is compatible with an alternative hypothesis for a physical function of caveolae, and this is the focus of the present report.

One of the initial mechanisms for mechanotransduction in response to fluid shear stress is the activation/deactivation of specific receptors (such as G-protein coupled receptors) located in the plasma membrane (Makino et al. 2006; Chachisvilis et al. 2006). The actions on different membrane receptors may be one of the reasons for the highly cell-specific responses observed among different cell types in response to fluid shear stress. Physiological fluid shear stress (e.g., 12 dyn/cm^2) in endothelial cells induces rapid tyrosine phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR2 or flk1), followed by clustering and subsequent Shc association (Chen et al. 1999). Shear stress also causes rapid activation of $\alpha_v\beta_3$ integrins (Shyy and Chien 1997; Jalali et al. 2001; Tzima et al. 2001), with increased binding to extracellular matrix proteins. In addition, the lipid membrane properties, which determine the mobility of membrane receptors, directly influence the fluid shear stress response (Park et al. 1998; Butler et al. 2002). Thus, receptors may serve as signal sensors and transducers across the membrane and respond not only to ligand binding, but also to the mechanical influence of fluid shear. However, it is unclear how endothelial cells that are constantly exposed to a vascular flow environment may undergo chemical activation from ligands if the receptors of these ligands on the cell surface are shear sensitive and may undergo constant stimulation by the flow environment.

We hypothesize the existence of a mechanism that distinguishes between the receptor activation induced by chemical agonists and by fluid shear stress. With the use of finite element analysis, we show in this report that the specialized membrane pockets formed by caveolae dramatically reduce the fluid shear stress in their interior and may therefore serve as fluid *shear stress shelters* for receptors in their interior. A receptor inside a caveola is protected from fluid shear stress stimulation, while it can remain responsive to an agonist that can diffuse through the open orifice of a caveola. In contrast, a receptor that is located in the outer membrane domain outside a caveola is exposed to fluid shear stress, and the shear activation or deactivation of the receptor may alter its response to its agonist. We explore this problem from a fluid mechanics point of view, by studying receptor activation inside and outside of caveolae by fluid shear stress and by receptor agonists. Furthermore, we provide experimental evidence for flk1 that is consistent with predictions from our mathematical analyses, suggesting that caveolae are fluid shear stress shelters.

2 Analysis and methods

2.1 Fluid shear stress and diffusion inside a caveola

The fluid flow in the vicinity of caveolae, which are small membrane structures (~0.1 μm in diameter), has a low Reynolds number (~0.001) with negligible inertial forces. The dimensions of blood cells are far too large to enter caveolae, and therefore, the fluid filling a caveola consists of plasma with a Newtonian viscosity ($\mu \sim 1.0$ cpoise). The flow of plasma at this scale is determined by the Stokes approximation of the equation of motion for an incompressible fluid with constant viscosity:

$$0 = -\overrightarrow{\nabla} p + \mu \nabla^2 \overrightarrow{v} \quad \text{and} \quad \nabla \overrightarrow{v} = 0, \tag{1}$$

where $\overrightarrow{\nabla}$ is the pressure gradient, $\overrightarrow{v}$ the fluid velocity vector and ∇^2 the delta operator. The left-hand side of Eq. 1 represents conservation of momentum and the right-hand side the incompressibility condition for plasma.

To determine the rate of diffusion in the presence of a convective/diffusive flow field in the vicinity of a caveola, we determined the concentration, c, of an agonist with diffusion coefficient, D, in plasma according to the flux, J:

$$\vec{J} = -D\vec{\nabla}c + c\vec{v}, \tag{2}$$

where $\overrightarrow{v}$ is the velocity vector computed according to Eq. 1. In the absence of significant alterations in agonist concentration (due to either new generation or enzymatic degradation) in the vicinity of a caveola, conservation of mass requires that

$$\frac{\partial c}{\partial t} = \vec{\nabla}\vec{J}. \tag{3}$$

We showed previously that the typical shapes of caveolae can be determined from membrane mechanics (Kosawada et al. 1999). We assumed membrane properties with bending and in-plane membrane shear strain energy. The flask-like shape of caveola readily forms as a result of mechanically linking an *inner* spherically curved membrane domain inside the caveola and an *outer* membrane domain (see Fig. 1). We also assumed that the inner membrane has a spherical (unstressed) resting shape with a spontaneous curvature ($1/r_\mathrm{I}$) produced by caveolin and the outer membrane has a nearly flat resting shape with only a small curvature ($1/r_\mathrm{O}$) (see Figure 2 in Kosawada et al. 2005). Coupling these two membrane domains at the neck gives the characteristic flask shapes of caveolae that are virtually identical to

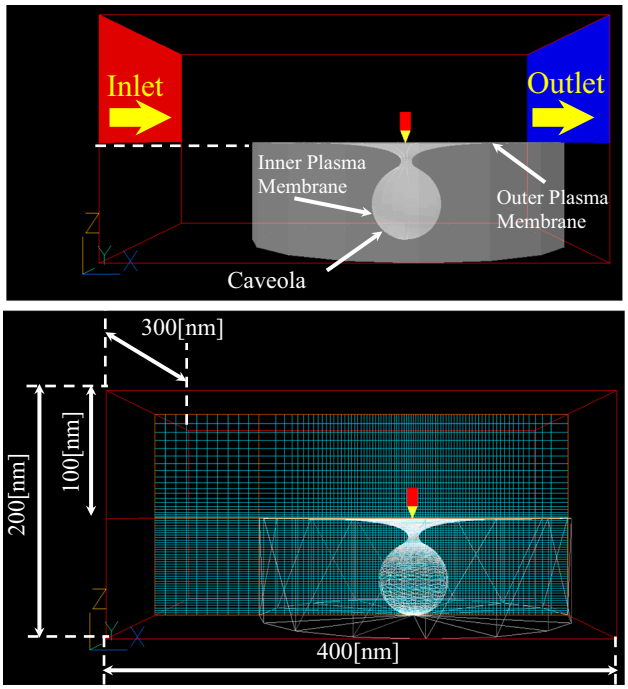

Fig. 1 Three-dimensional flow domain over a caveolae (shown for the case of $n = 100$, $r_t = 4.451 r_I$ in reference Kosawada et al. 2005). The number of elements in the flow domain is 90, 80 and 60 in X, Y and Z, directions, respectively. The inlet mean velocity U is set as 0.051 mm/s

the shapes seen with electron microscopy (Kosawada et al. 2005).

We solved Eqs. 1–3 in a cubic domain around the caveola in accordance with the following boundary conditions: The plasma velocity is zero (due to no slip) on the plasma membrane surface everywhere inside and outside the caveola. On the plane upstream of the domain (~4 caveolar diameters from the neck), we applied a linear velocity field with a typical wall shear rate in the circulation (~416 s^{-1}). On the plane above the caveola (parallel to the outer membrane) and the two planes parallel to the flow direction to the right and left of the cell, we assumed zero normal velocity to those planes (Fig. 1).

In the case of agonist diffusion, we assumed that at an initial time ($t = 0$) on the plane upstream of the caveola, there is instantaneously a normalized agonist concentration $c_0 = 1$, with zero concentrations elsewhere in the flow field. We assumed the diffusion coefficient D to be 1.26×10^{-5} cm^2/s. Based on these assumptions, we determined the rate at which the agonist is transported into the caveola. We assumed the membrane in and around the caveola is impermeable to the agonist, so that there is no flux of the agonist across the plasma membrane. The velocity field ($\vec{v}$) around the caveola in Eq. 2 was computed according to Eq. 1.

The solutions to these boundary value problems were obtained numerically with a finite element analysis (FEM, Phoenics, 3.6 version, CHAM., Ltd., Wimbledon, London, UK). In this analysis, we set the dimensions of the flow domain to be $90 \times 80 \times 60$ (length, width, height directions, respectively) with a total of 432,000 elements. Depending on each particular membrane model, we adjusted the number of elements and nodes and set the diameter of the undeformed inner membrane domain (a spherical vesicle) at $2r_I =$ 66.7 nm. For the dimensions of the plasma flow domain above the outer membrane domain, we selected a width, height and length of 300, 100 and 400 nm, respectively (Fig. 1). We simulated a flow profile for a complete blood vessel model (inner diameter 0.006 mm) with a mean velocity of 0.35 mm/s. From these values, we estimated the inlet mean velocity parallel to the outer membrane to be 0.051 mm/s in the computational domain (Fig. 1) over the caveola.

The shear stress distribution on the luminal surface was computed as the wall shear stress based on "log-law wall functions." It is friction velocity squared, equivalent to shear stress divided by plasma density (with dimension cm^2/s^2).

2.2 Cell culture

Primary cultures of bovine aortic endothelial cells (BAEC) were harvested from bovine aorta after collagenase digestion as previously described (Shiu et al. 2004). BAEC were subcultured in Dulbecco's modified Eagle growth medium containing 10% fetal bovine serum, 1% sodium pyruvate, 5 U/mL heparin, 100 mM L-glutamine and 1% penicillin–streptomycin and maintained at 37 °C in a humidified incubator with 5% CO_2–95% air. In preparation for in vitro shear experiments, BAEC (passages 5–10) were seeded on glass slides and cultured for 1 day. At that time, these cell preparations were assembled into a parallel plate flow system. All cell culture reagents were purchased from Gibco BRL (Carlsbad, CA).

2.3 Fluid flow chamber

We used a parallel plate flow system to expose BAEC to 12 dynes/cm^2 fluid shear stress as previously described (Frangos et al. 1985). During the flow experiments, the system was maintained at 37 °C in a constant temperature hood, and the circulating DMEM with 10% FBS was ventilated with humidified 5% CO_2–95% air mixture.

To examine the role of caveolae structures in tyrosine phosphorylation of VEGFR-2 by VEGF (VEGF-A or VEGF165), BAEC were maintained under control conditions in either the presence or the absence of 25 ng/mL human VEGF.

2.4 Immunoprecipitation and immunoblotting

BAEC lysates were harvested in lysis buffer (containing 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS and 1% Triton X-100) and clarified by centrifugation. Aliquots of BAEC extracts containing equal amounts of protein were then immunoprecipitated (IP) with a polyclonal antibody specific for caveolin-1 at 4 °C for 2 h. The resulting antibody–protein complexes were separated according to molecular weight using SDS-PAGE and transferred to a nitrocellulose membrane. These membranes were probed with polyclonal antibodies to either VEGFR-2 (Upstate Biotech, Charlottesville, VA), pVEGFR-2 (Santa Cruz Biotech, Santa Cruz, CA) or caveolin-1 (Santa Cruz Biotech, Santa Cruz, CA) using standard immunoblotting techniques. Proteins of interest were visualized using the appropriate secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) and an ECL detection system (Amersham, Arlington Heights, IL). In some cases, the supernatants from immunoprecipitation procedure were saved and subjected to a second IP using the polyclonal antibody for VEGFR-2 and analyzed as described above.

2.5 Immunofluorescence labeling and detection of caveolae and membrane receptors

BAEC on borosilicate glass substrates were fixed with 2% *p*-formaldehyde (Fisher Scientific, Pittsburgh, PA) in phosphate-buffered saline (PBS) and permeabilized with 0.1% triton X-100 (Sigma, St. Louis, MO) in PBS. The cells were dual-labeled with polyclonal rabbit anti-caveolin-1/TRITC conjugates (clone N-20, Santa Cruz Biotechnologies, Santa Cruz, CA) and polyclonal goat antibodies to phosphorylated VEGFR-2 (Santa Cruz Biotechnologies). The membrane receptor–antibody complexes on the cell layers were then fluorescently labeled with donkey anti-goat IgG-Alexa-488. Stained cell preparations were mounted using Vectashield (Vector Laboratories, Burlingame, CA) and visualized using a rotating-disk laser confocal microscope (Olympus, Melville, NY) with either fluorescein isothiocyanate (490-nm excitation/520-nm emission) or rhodamine (554-nm excitation/572-nm emission) optics.

Confocal images were stored (as TIFF files) and processed digitally (NIH Image, Scion Corporation, Frederick, Maryland) to examine co-localization of caveolin-1 with the membrane receptor of interest on the cell surface, but not to compare protein levels of these molecules between experimental treatments. The average percentage of pVEGFR-2 that co-localized with caveolin-1 was measured from at least 20 discrete membrane regions derived from 2 to 3 optical fields in each of three or more independent experiments.

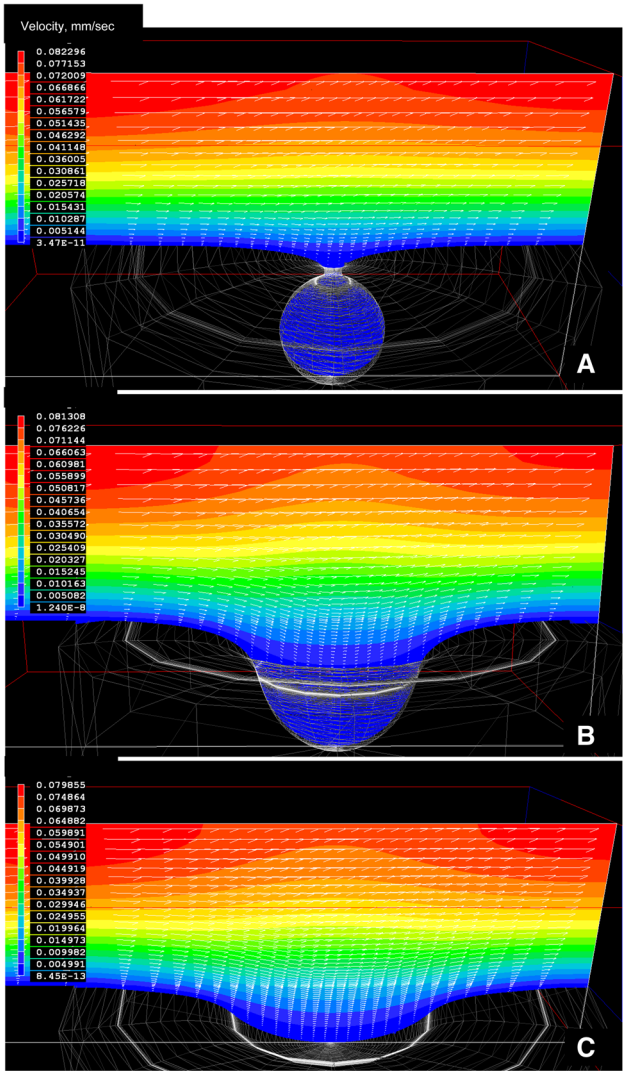

Fig. 2 Velocity vectors and velocity contours around a caveolae with **A** typical neck, **B** partially opened and **C** a caveolae that has been opened by membrane tension. Case A corresponds to the spontaneous radius $n = 100$, $r_t = 4.412 r_I$, case B corresponds to $n = 10$, $r_t = 4.556 r_I$, and case C to $n = 10$, $r_t = 4.805 r_I$ in Figure 4 of Kosawada et al. (2005). The color code for the velocities is in units of mm/s. For clarity, the velocity vectors are displayed only in the midplane of the caveolae. The membrane of the caveolae is shown by means of the white colored grid lines. Note the low velocities inside the caveolae (**A**)

3 Results

3.1 Flow velocities, membrane shear stresses, but not fluid pressure, are reduced inside caveolae

The velocities inside a caveola are significantly lower than those on the outer membrane domain (Fig. 2A). This is a consequence of the neck region of the caveola with a rapid transition of the velocity from magnitudes in the range of 0.05 mm/s on the outer domain to much lower values in

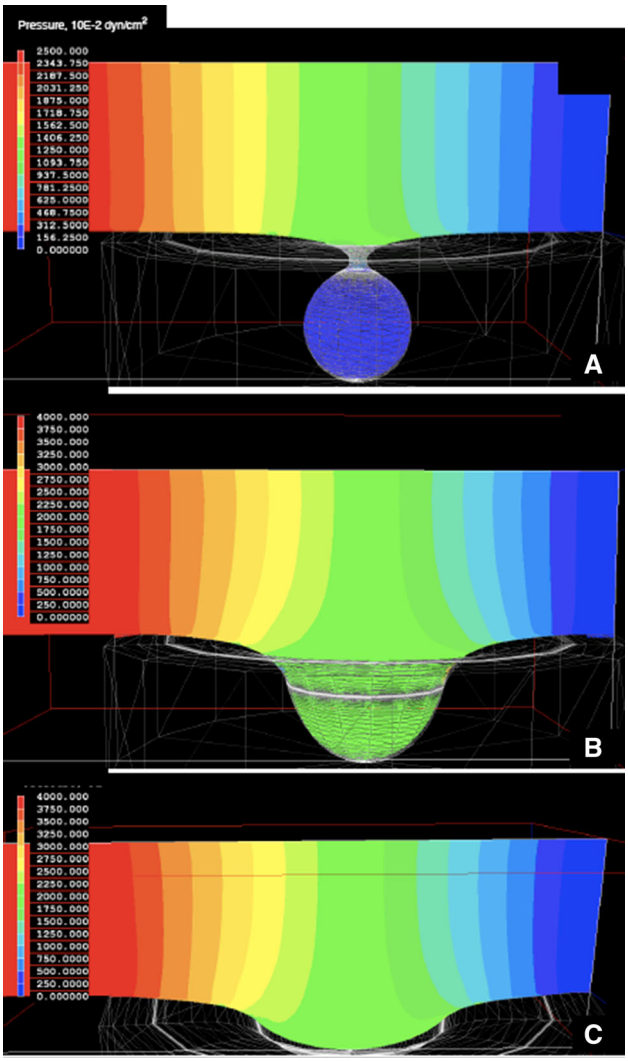

Fig. 3 Fluid pressure distribution in and around a caveolae. The membrane shapes are the same as shown in Fig. 1, with a caveola before (case A), after partial (case B) and almost complete membrane unfolding (case C). The color code for the pressures is shown (left) in units of 10^{-2} dyn/cm^2

the interior of the caveola. As a caveola is pulled open by membrane tension (Fig. 2B), the neck region becomes less effective in reducing the velocity in the vessel lumen, thus enhancing the convective transport in the caveola. If the membrane tension is further increased, the membrane starts to flatten further—now reduced to a membrane indentation—and consequently the fluid shear rate on the inner membrane of a caveola increases further and assumes the same value as that on the outer membrane.

In contrast to effects of the caveolar geometry on the velocity field, the plasma pressure inside the caveola is almost identical to that present at the same axial location along the vessel lumen (Fig. 3A). The pressure gradient in the outer fluid domain over the dimension of the neck of a partially opened caveola is small (due to the small size of the caveola) even though it increases as the neck region is stretched (Fig. 3B, C). The intra-caveolar pressure is similar to the plasma fluid pressure outside the caveola due to the small pressure gradients across the neck of the caveola and inside it.

The inner membrane surface of a caveola with a neck region is subject to a dramatic reduction in the fluid shear stress (Fig. 4A), falling at the bottom (position f in Fig. 4A) to about 10^{-5} of the fluid shear stress on the outer membrane. In line with the velocity, the shear stress inside a caveola depends on the degree of membrane narrowing in the neck region. The fluid shear stress inside the caveola remains reduced even if the membrane neck region is partially unfolded (Fig. 4B), but increases as the membrane is stretched and the caveola reduced to an indentation (Fig. 4C).

The caveolar structure permits rapid diffusion of an agonist through its open neck into its interior (Fig. 5) in the absence of any structure that blocks the opening. An agonist that is carried in the blood stream largely by convection from an upstream location to the neck region of the caveola encounters resistance to convective transport into the interior of the caveola (e.g., see frames between 1 and 24 ms in Figs. 5, 6). At that instant of time, however, the agonist is rapidly carried by diffusion across the neck into the interior of a caveola; within a few milliseconds (frames between 24 and 40 ms of Fig. 5), the agonist concentration inside the caveola of ~0.013 rises to a value of 1 (the normalized outside concentration) due to the relatively small dimensions of caveolae (Fig. 6). The results in Figs. 5 and 6 were obtained by assuming unimpaired diffusion across the neck. In the presence of restricted diffusion in the neck or inside the caveolae, the agonist entry time would be prolonged (see Sect. 4).

3.2 Fluid shear stress increases levels of pVEGFR-2, but not its co-precipitation with caveolin-1

We examined shear-activated VEGFR-2 (Wang et al. 2002) and its association with caveolin-1 in BAEC. Cells either maintained under control condition (no-flow) or exposed to 12 dynes/cm^2 fluid shear stress for 5 min exhibited similar levels of pVEGFR-2 that co-immunoprecipitated with caveolin-1 (Fig. 7A). In contrast, compared to controls there was an increased amount of pVEGFR-2 that did not co-immunoprecipitate with caveolin-1 in BAEC exposed to shear stress for 5 min (Fig. 7B). These results indicate that shear stress activates VEGFR-2 located outside the caveolar domains of the cell surface.

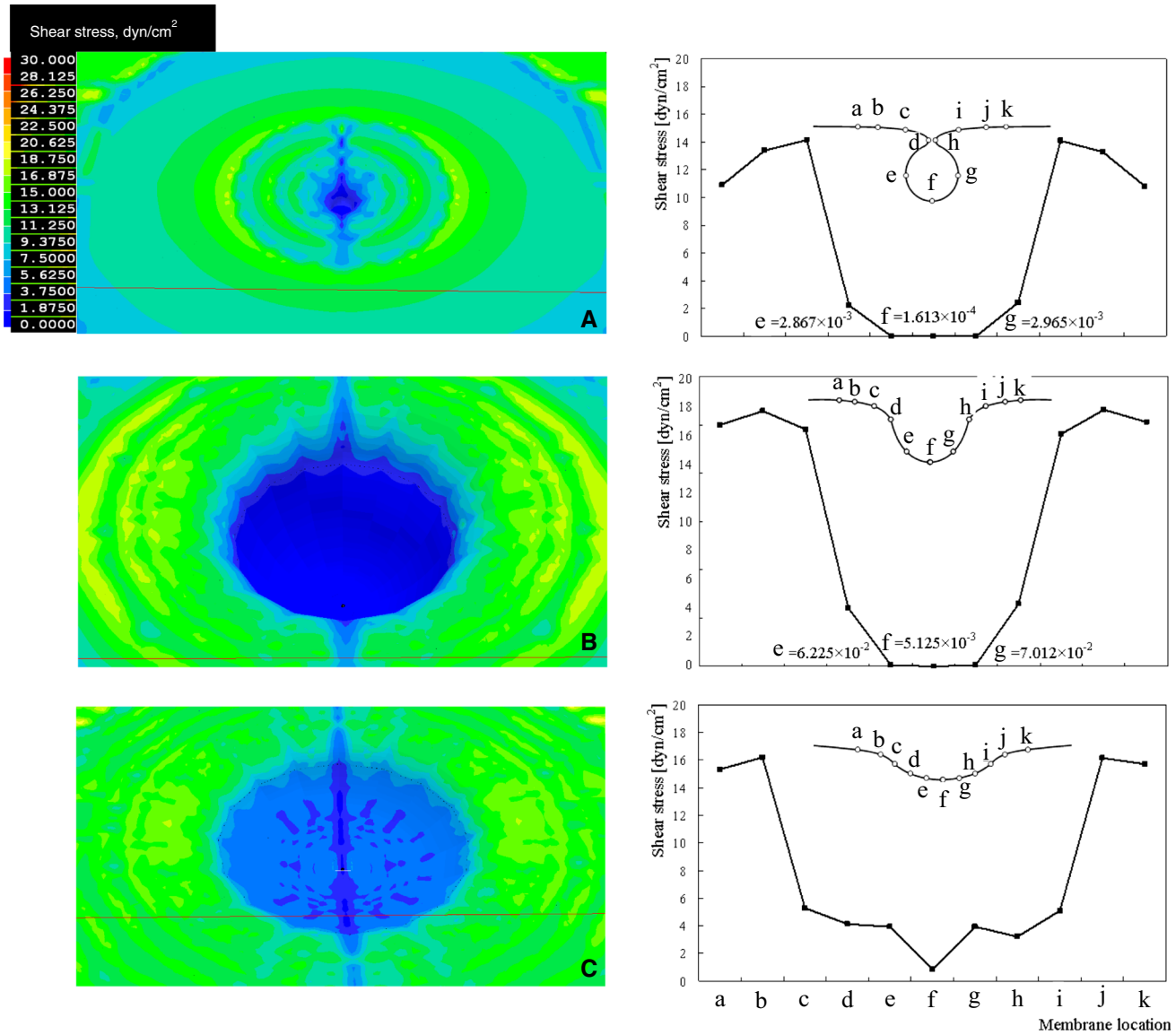

Fig. 4 Topical view (left panels) and distribution profile (right line graph) of the shear stress on the outside and inside membrane surface of a caveolae. The color code for the shear stresses (left) is in units of dyn/cm^2. The case shown is the same as for the membrane shapes in Fig. 1, with **A** an intact caveola with neck region, **B** a partially unfolding caveola and **C** after almost complete unfolding of the caveola

3.3 VEGF stimulation, but not shear stress exposure, increases co-localization of VEGFR-2 with caveolin-1 on the endothelial membrane

Confocal analyses reveal a punctate localization of pVEGFR-2 (green staining in Fig. 8) and caveolin-1 (red staining in Fig. 8) on BAEC. The fluorescence is associated with the membrane within the resolution of confocal microscopy and observed either under no-flow condition (control), after exposure to 12 dynes/cm^2 fluid shear stress, or after stimulation with 25 ng/mL VEGF for 5 min (Fig. 8). Compared to respective controls, stimulation of BAEC with VEGF (25 ng/mL), but not with fluid shear stress for 5 min, resulted in a significant ($p < 0.05$) increase in the co-localization of pVEGFR-2 with membrane-bound caveolin-1 (Fig. 8).

4 Discussion

The results of the current study suggest that caveolae may serve as an ideal structure to prevent exposure of mechanosensitive membrane receptors to fluid shear stress in the blood stream. The caveolar neck region restricts expo-

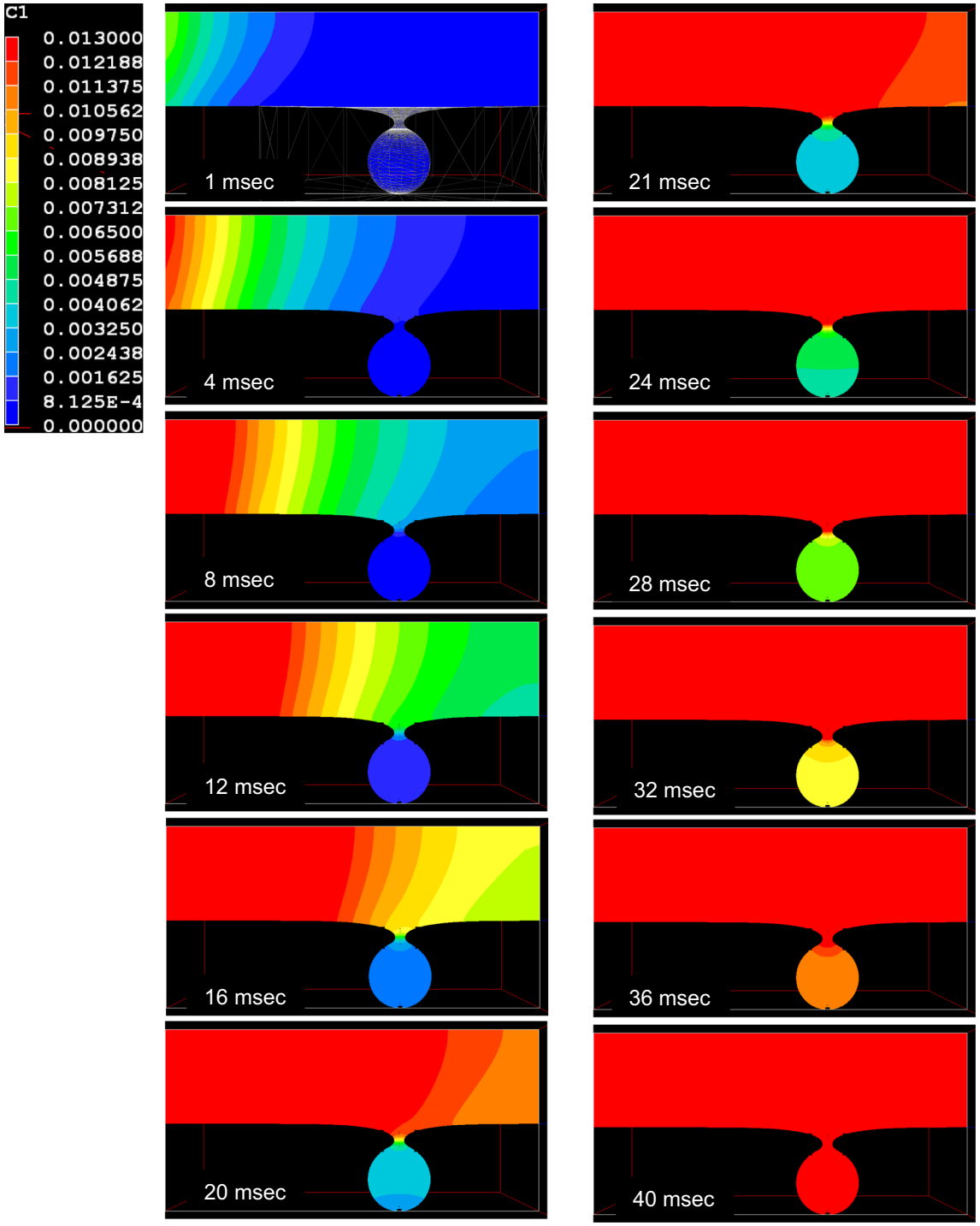

Fig. 5 Agonist concentration profile over a caveolae (case A in Figs. 2, 3, 4), which is released from the left side into the plasma flow. The color code for the fractional mass concentration (C1), which is normalized by the concentration at the time of 40 ms where the concentration at the bottom of the caveolae reached 1.3% compared to the one at the inlet, is shown on the left

sure of the plasma membrane inside the caveolae to the fluid flow on the outer membrane, thereby minimizing the fluid velocity inside the caveola. At the same time, the small size of the caveola permits rapid diffusive transport of plasma proteins/peptides (i.e., ligands) into the interior, where a variety of membrane receptors are located (Schnitzer et al. 1995; Zhu and Smart 2003; Pike 2005; Chini and Parenti 2004).

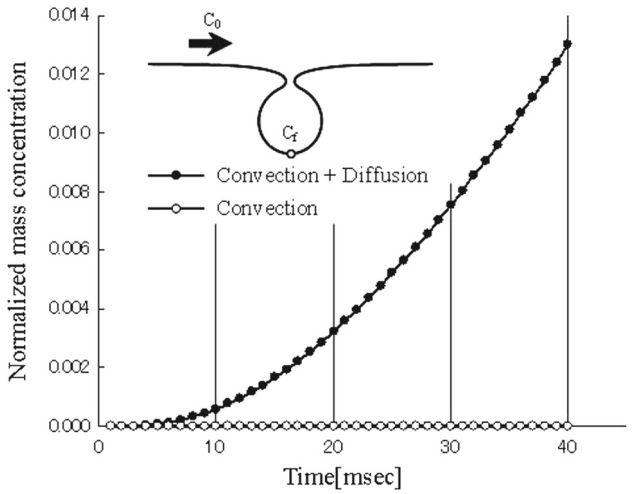

Fig. 6 Normalized agonist concentration at the bottom of the caveolae (c_f, at point f) with an applied concentration ($c_0 = 1$) during transport in the blood stream by convection and diffusion according to Eq. 2 (full circles) (same as in Fig. 5). For comparison, the concentration c_f is shown in the case of convection only without diffusion (open circles)

The particular flask-like shape of caveolae likely forms as a consequence of the attachment of two membrane domains with different resting stress-free membrane shapes, an inner curved membrane with spontaneous curvature (possibly coated with caveolin) and an outer flat membrane without caveolin. Assuming such resting shapes for the membrane, the flask-like shape can arise assuming pure membrane bending energy or a combination of bending and in-plane shear deformation energy (Kosawada et al. 2005). Chained vesicles also assume a similar configuration of membranes with neck regions and can form on the basis of the same fundamental assumptions (Kosawada et al. 1999, 2001; Kosawada and Matsukawa 2003). Thus, a critical issue in the generation of a caveola is the presence of an inner curved membrane with an asymmetric lipid bilayer in which one leaflet is expanded, a function that may well be recognized by the asymmetric positioning of caveolin in the double lipid layer (Sternberg and Schmid 1999).

We assumed in the current analysis that transport in the plasma by diffusion is unrestricted across the neck and inside the caveola (Figs. 5, 6). Consequently, caveolae may rapidly fill with any molecule in plasma that is adjacent to the endothelial membrane. However, the presence of a diaphragm (Stan et al. 1999a) in form of the protein PV-1 in the neck of a caveola (Stan et al. 1999b) may act as a molecular sieve to hinder or even block the entry of larger plasma proteins into the interior of the caveola.

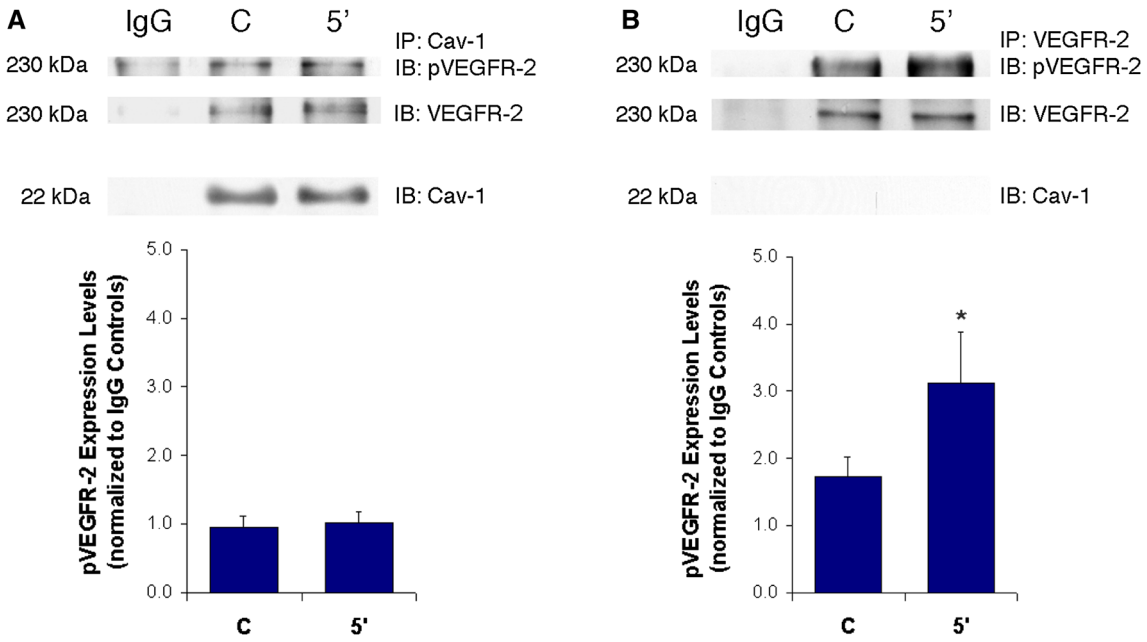

Fig. 7 Cellular lysates of BAEC either maintained under no-flow conditions (*C*) or exposed to 12 dynes/cm^2 fluid shear stress for 5 min were immunoprecipitated (IP) with a polyclonal antibody for caveolin-1 (Cav-1, **A**). The immunoprecipitates were electrophoresed and immunoblotted (IB) for pVEGFR-2. Similar levels of pVEGFR-2 were found to be associated with caveolin-1. Both VEGFR-2 and caveolin-1 were immunoblotted to demonstrate equal lane loading (bottom of **A**). When the same cell lysates were immunoprecipitated a consecutive second time with VEGFR-2 antibodies (Panel B), lysates of BAEC exposed to shear stress for 5 min contained increased amounts of pVEGFR-2 that was not associated with caveolin-1 as demonstrated by the absence of caveolin-1 in the immunoprecipitates (bottom of **B**). Bar graphs are mean ± SD band densities of pVEGFR-2/VEGFR-2 and normalized by the band density of the IgG ($n = 3$). IgG denotes the use of a negative antibody control for the immunoprecipitations. *$p < 0.05$ compared to control by Student's *t* test

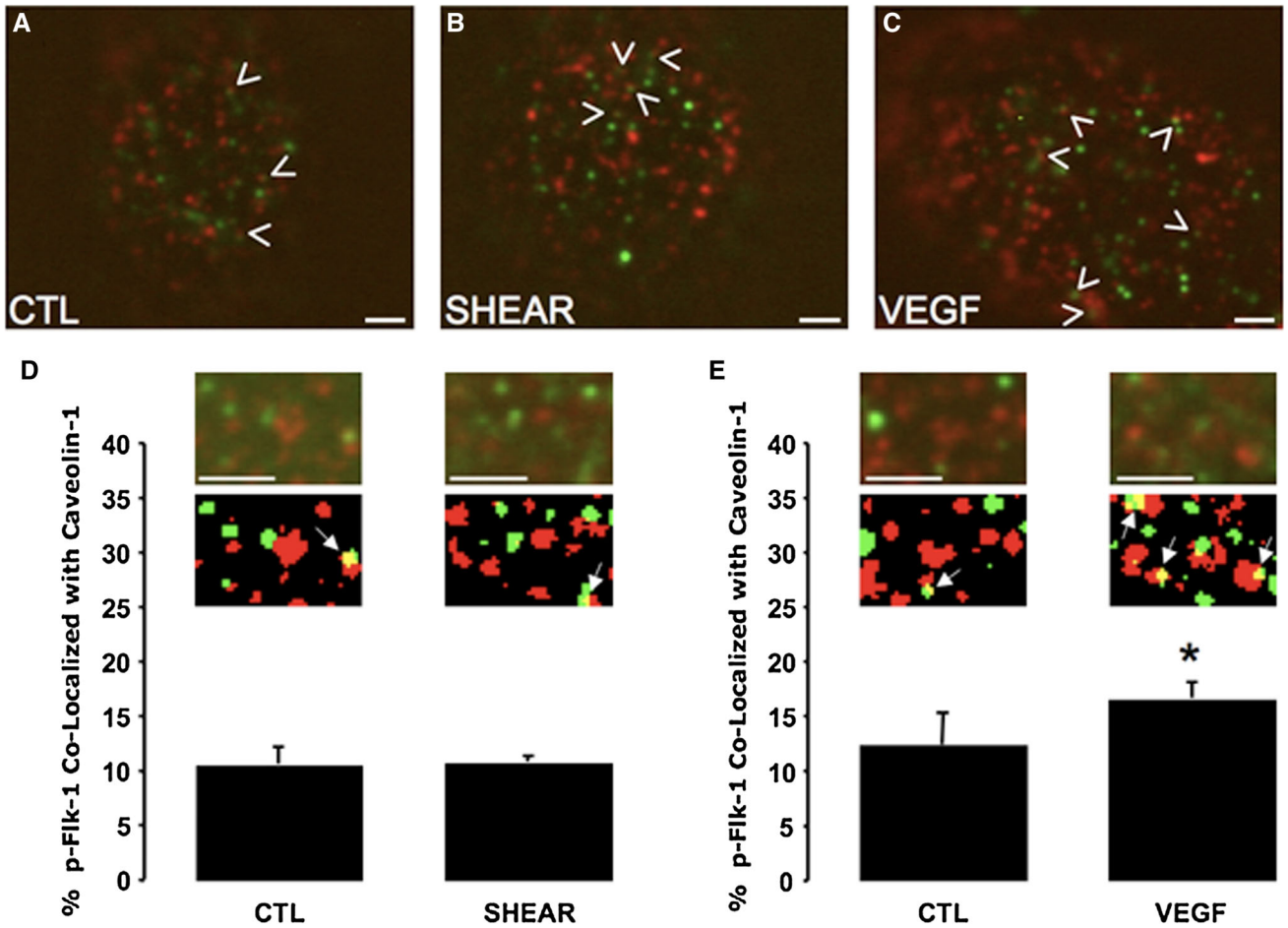

Fig. 8 Regions of the plasmalemmal membrane on the apical surfaces of BAEC either maintained under static no-flow conditions (**A**, CTL), exposed to 12 dynes/cm^2 shear stress (**B**, SHEAR) or stimulated with 25 ng/mL VEGF (**C**, VEGF) for 5 min exhibited punctate staining for caveolin-1 (red) and pVEGFR-2 (green) as visualized by confocal immunofluorescence. Under all conditions tested, a percentage of pVEGFR-2 co-localized with caveolin-1 (yellow regions in **A**–**C**, some indicated by the arrowheads) as assessed by thresholding analyses of discrete regions of endothelial surfaces derived from confocal micrographs of cells (inset images in **D** and **E**, yellow regions indicated by small arrows). BAEC either maintained under control conditions as well as cells exposed to fluid shear for 5 min exhibited similar percentages of pVEGR-2 that co-localized with caveolin-1 (**D**). The percentage of pVEGFR-2 co-localized to caveolin-1, however, was significantly increased on VEGF-stimulated BAEC relative to controls (**E**). White horizontal bars in confocal micrographs presented in **A**–**E** = 2 μm. Vertical bars in **D** and **E** are mean ± SEM; n = at least 3 separate experiments. *$p < 0.05$ compared to control by Student's t test

In addition, the molecular sieve may also serve to further reduce the velocity in the interior of a caveola, below the values in the current results derived without such a sieve. Thus, the particular shape of caveolae may permit selective binding of agonists to receptors in their interior, while they can effectively shelter the same receptors from the effects of fluid shear stress. This may be an important mechanism from two points of view. Since a receptor that has been activated by fluid shear may become unresponsive to agonist binding, caveolae may serve as a mechanism to assure that receptors in their interior can be activated by agonists without pre-activation by fluid shear. Furthermore, in the presence of continuous free fluid motion in the blood stream, the encounter and binding of a soluble agonist and its receptor is under the control of fluid shear stress, a process that may reduce the binding times and may favor dissociation. Caveolae may serve as a reservoir for agonist–receptor interaction to ensure the signaling. In addition, caveolae also serve as a membrane reservoir for shear-sensitive signaling if the plasma membrane is being stretched (Schmid-Schönbein et al. 1995; Lee 1990). Thus, caveolae may serve multiple functions in receptor regulation.

The presence of caveolae in many cells—other than just endothelial cells—indicates that basic physiological functions are being fulfilled by these structures, above and beyond the transcytotic transport process assumed in the past. While there is evidence that caveolae have the ability to transport ligands into peri-nuclear compartments (Handley and Chien 1987; Chien et al. 1982; Anderson 1993),

there is less evidence that these membrane structures act as a shuttle mechanism across non-activated endothelium or in connective tissue cells that also have caveolae. Most caveolar/vesicular profiles are attached to the plasma membrane even if they appear as isolated vesicle membranes in the interior of the cell cytoplasm, as shown by labeling studies after cell fixation (Chien et al. 1982). Caveolae made visible under living conditions with fluorescent proteins show little movement as seen by confocal microscopy (Thomsen et al. 2002). The current hypothesis of caveolae as shelters for fluid shear stress is compatible with the fact that many cell types have caveolae even if located in the interstitial space (Lorenzen-Schmidt et al. 2006; Garanich et al. 2005; Johnson et al. 1996) where they are also exposed to fluid shear stress (Tada and Tarbell 2002; Hillsley and Frangos 1994).

Evidence from studies (Woodman et al. 2003) involving endothelium from caveolin-1(–/–) mice supports the role of caveolae as membrane structures that may regulate activation of angiogenic growth factor receptors (such as flk-1) under the influence of either ligand binding or shear stress. The fact that these caveolin-1-deficient mice exhibit reduced numbers of caveolae in parallel with a reduced ability for capillary angiogenesis (Woodman et al. 2003) is compatible with loss of their ability to differentiate between an agonist (i.e., VEGF) and a shear stress mediated mechanism. Caveolin-1-deficient mice also have an "over-stimulated" endothelium as indicated by constitutive e-NOS activation (Schubert et al. 2002), which may be due to the lack of caveolar shelters that would protect the e-NOS from shear stress exposure. The loss of caveolae may therefore cause mechanosensitive receptors to be constantly exposed to shear, leading to receptor pre-activation and overstimulation by fluid shear stress. Consequently, constant exposure to fluid flow would attenuate the ability of endothelial cells to respond to agonist stimulation.

The significance of caveolae as shear stress shelters is revealed in studies utilizing mice with caveolin gene deletions without caveolae in specific organs or cells. Besides overstimulation of signaling pathways without caveolae, they suggest that deletion of caveolin reduces the ability of endothelial cells to orient in fluid shear stress (Yang et al. 2011) and attenuation of a fluid shear stress stimulation by caveolae may be required for development of a normal, well-oriented heart fiber structure (Park et al. 2002).

One family of receptors thought to play a role in mechanotransduction is tyrosine kinase growth factor receptors. Previous reports demonstrate tyrosine phosphorylation of flk-1 and tie-2 upon exposure of BAEC to fluid shear stresses (Wang et al. 2002; Lee and Koh 2003). Moreover, these receptors are present on the cell surface both inside and outside caveolae. In the present study, we provided evidence (Figs. 7, 8) that, upon either mechanical (shear) or chemical (VEGF) stimulation, phosphorylated flk-1 receptor is present inside caveolae (as indicated by their close association with caveolin-1) as well as outside these membrane invaginations. Under shear stimulation, however, phosphorylated flk-1 is preferentially located outside caveolae consistent with our mathematical model. Our model predicts the internal surfaces of caveolae to be subjected to low shear magnitudes (Fig. 4) that may be below the threshold levels necessary to activate flk-1. In contrast, stimulation of cells with VEGF results in increased amounts of phosphorylated flk-1 receptor outside as well as inside caveolae (Figs. 7, 8), which agrees with our predictions for rapid diffusion of ligands into the caveolar invaginations.

Rizzo and his colleagues designated the caveolae as mechano-sensor centers (Rizzo et al. 1998b) due to the large number of receptors inside the caveolae and the observation of significant e-NOS activation in isolated caveolae. On the surface, this view appears to contradict the current hypothesis. However, it is important to point out that those investigators used a 1-min perfusion of the lung before isolation of the caveolae by the silica coating technique (Jacobson et al. 1992). They carefully checked that the enhanced e-NOS activity is not the result of additional e-NOS being recruited to the plasma membrane during the 1-min perfusion. Perfusion of a living blood vessel in an organ, such as the lung, is associated with a pressure-dependent distension of microvessels and their endothelial components (Fung and Sobin 1972; Yen 1989), thus potentially causing the unfolding of caveolae (Lee and Schmid-Schönbein 1995) and exposing membrane receptors (e.g., e-NOS) to fluid shear stress in the (micro)vascular lumens. In contrast, the current observations in intact endothelial cells on a glass slide without significant cell distension show low levels of receptor activation under fluid shear if the receptors are located inside the caveolae. The role of caveolae in signaling under conditions that can cause cells to be stretched or relaxed and caveolae to be unfolded or folded requires further investigations with careful control of the caveola density and shapes during fluid flow, since the details of their shapes determine the fluid shear stress on the plasma membrane.

An interesting question arises from the current proposal of shear stress shelter whether activation of a receptor, like VEGFR2, by fluid shear stress is different from its activation by VEGF. Whereas there may be distinct differences in the time course of these two stimulants, the conformational changes in the receptor associated with a fluid shear stress as compared with binding of a ligand remain to be elucidated by molecular simulation studies. The two different forms of receptor stimulations could lead to differences in intracellular signaling.

In conclusion, caveolae, besides their classical role as transport and signaling membrane structures, may also play a role in the differential regulation of membrane receptor activation by chemical ligands and fluid shear stress.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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