Shear-Induced Dissociation of Gq-PECAM-1 Complexes Localized to the Endothelial Cell-Cell Junction.

Loufrani: **Gq-PECAM-1 Co-localization and Fluid Shear**

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ABSTRACT

As the inner lining of the vessel wall, vascular endothelial cells are poised to act as a signal transduction interface for hemodynamic forces. The molecular mechanisms of the primary force sensing elements of the cell, and the cascade of events that are involved in the mechanochemical signal pathway remain unclear. Activation of the G protein Gαq and the phosphorylation of platelet endothelial cell adhesion molecule-1 (PECAM-1) have been reported to be involved in the early events of shear-induced signaling pathways. Using immunofluorescence and co-immunoprecipitation techniques, we demonstrate here for the first time that Gαq and PECAM-1 co-localize as a complex at the cell-cell junction in primary human umbilical vein endothelial cells. Temporal gradients in shear stress lead to a rapid disassociation and re-association of the Gαq-PECAM-1 complex within 15 sec (0.65 ±0.059 ratio Gαq/PECAM-1 versus sham control), whereas slowly transitioning fluid flow devoid of temporal gradients does not disrupt the complex (1.04 ±0.08 ratio Gq/PECAM-1 versus Sham control). Inhibition of protein kinases and tyrosine kinases completely eliminated impulse flow induced Gαq-PECAM-1 disassociation and PECAM-1 phosphorylation. Taken to together, this data may represent a missing link between the primary force sensing elements of the cell, and the downstream mechanochemical transduction pathway.

CONDENSED ABSTRACT

Cellular force sensing elements and mechanochemical signal pathways are unclear. Gq and PECAM-1 are reported involved. Gq and PECAM-1 was co-localized as a complex at the cell-cell junction in HUVEC. Shear stress leads to a rapid disassociation of the complex. A link between force sensing and downstream pathways are suggested.

KEY WORDS

shear stress; platelet endothelial cell adhesion molecule-1 (PECAM-1); G proteins; human umbilical vein endothelial cell (HUVEC); cell-cell junction

INTRODUCTION

Atherosclerosis remains a leading cause of morbidity and mortality in the Western world **(1)**. It is a chronic systemic disease attributed to many well-identified risk factors (i.e. diabetes, hypercholesteremia, and cigarette smoking). Yet the formation of atherosclerotic lesions do not occur in a random fashion. Detailed analyses of fluid mechanics in atherosclerosis-susceptible vascular regions reveal a strong correlation between endothelial cell dysfunction and areas of low mean wall shear stress and large temporal gradients (flow reciculation and oscillation). Conversely, in regions predominated by steady unidirectional flow, where mean wall shear stress is high and temporal gradients are low, the occurrence of plaque formation is correspondingly very low **(2**). This pattern of plaque formation, independent of other associated risk factors, has lead a number of investigators to suggest that steady fluid flow within the vasculature is atheroprotective, whereas unsteady flow is pro-atherogenic **(3)**.

The molecular basis of shear-induced mechanochemical signal transduction, and the endothelium's ability to discriminate between flow profiles remains largely unclear. Given that fluid shear stress does not involve a traditional receptor / ligand interaction, identification of the molecule(s) responsible for sensing fluid flow and mechanical force discrimination has been difficult. It has been shown that the lipid bi-layer itself may act as a flow sensing receptor **(4)(5)**. Fluid shear stress is a frictional force that acts on the apical surface of the endothelial monolayer. Blood shear induces tensile stress across the endothelial cell membrane, which in turn resists the imposed shear with tension. Analysis of stress distribution in the endothelial cell membrane shows that tension is greatest within the cell-cell junction **(6).** *In vivo*, when an endothelial monolayer is exposed to increasing levels of fluid shear stress,

cell-cell junction-associated proteins have been shown to undergo significant dose dependent reorganization, whereas the integrity of the endothelial monolayer remains unaffected **(7)**.

A number of membrane associated proteins are specifically localized to the cell-cell junction. Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a cell adhesion molecule localized to the interendothelial cell-cell adhesion site. PECAM-1 (also known as CD31 or endoCAM) is the cell-cell adhesion molecule most abundantly expressed in endothelial cells. It is also expressed by platelets, monocytes, neutrophils, and a certain subset of T-lymphocytes **(8)**. In cultured endothelial cells, PECAM-1 is diffusely distributed in the plasma membrane of solitary cells, but once a cell-cell contact is made, it accumulates at the contact site. In endothelial cells forming a confluent monolayer, it is exclusively localized along the cell-cell border **(9)**. When confluent endothelial cells are exposed to physiological levels of fluid shear stress, PECAM-1 has been shown to be tyrosine-phosphorylated **(10)**. Other studies strongly suggest that mechanically induced tyrosine phosphorylation of PECAM-1, is not a downstream event of Ca2+ mobilization, K+ channel activation, stretch-activated cation channel activity, or PKC activation **(11)(12)**.

The rapid activation of heterotrimeric G proteins is known as an early flow mediated response in endothelial cells **(13)**, and may also play a role in the ability of the endothelium to discriminate between flow profiles. In confluent endothelial monolayers, the sudden temporal onset of flow induces a burst of NO production. This process is both calcium- and G-protein-dependent. In contrast, the prolonged steady shear stress that follows induces a sustained release of NO, at a rate of 10% of the initial peak of NO production, and is both calcium- and G-protein-independent

(14). This suggests that shear-induced stimulation of endothelial cells is derived from the superposition of two independent mechanical stimuli (steady shear and temporal changes in shear stress), which are in turn transduced by two different mechanochemical pathways **(15)**. Furthermore, membrane linked force transduction appears to be mediated at least in part, by the Gαq heterotrimeric G protein.

In the present study we test a comprehensive hypothesis on the mechanism of mechanochemical transduction in endothelial cells. This study is the first to demonstrate the co-localization and association of Gαq and PECAM-1 to the endothelial cell-cell junction. Furthermore, shear-induced disassociation of the Gαq-PECAM-1 complex suggests a possible shear sensing mechanotransduction pathway. We believe that this study helps to provide a fundamental understanding of how the endothelium senses hemodynamic forces in both normal physiology and vascular disease.

MATERIALS AND METHODS

Cell Culture and Shear Stress

Primary human umbilical vein endothelial cell (HUVEC) isolation was performed as previously described **(16)**. Cells were seeded onto glass microscope slides and grown to confluence within 4 days in M199 media (Irvine Scientific). Prior to all experimental procedures, the HUVEC were serum-starved for 4 hours to establish quiescence in the monolayer. For studies of PECAM-1 phosphorylation, cells were incubated for 30min in 50 μM H7 and genistein (wide spectrum protein kinases and tyrosine kinases inhibitors, respectively) immediately prior to experimental procedures **(17).** In a conventional parallel-plate flow chamber **(16)**, confluent HUVEC monolayers were subjected to one of the following flow profiles: **(1)** impulse (1 sec impulse of 14 dyne/cm2). **(2)** ramped-transient (a smooth 15 sec ramped increase from 0 to 14 dyne/cm2, sustained for 1 sec, followed by a 15 sec ramped decrease) **(Figure 1)**. All flow chambers and accompanying apparatus were maintained at 37^0C throughout the experiment. Perfusing medium was driven via a computer-controlled syringe pump. HUVEC were harvested 15s, 30s or 3min after the cessation of each specific flow profile. Cells were quickly transferred to ice-cold buffer and lysed (see below). Time-matched sham controls (slides mounted on chamber without flow) were performed for all experimental procedures.

HUVEC Confocal Immunofluorescence Studies

All reactions were performed at room temperature. HUVECs on glass slides were gently washed with ice-cold phosphate-buffered saline (PBS) and fixed for 45min in 4% paraformaldehyde. Fixed cells were rinsed with 25mM NH4Cl to quench free aldehyde groups. Cells were then permeabilized with 0.1% Triton X-100

for 10min. For double labeling HUVECs were then successively incubated with: i) 5% rabbit or goat normal serum to block for non-specific staining. ii_a) goat anti-PECAM-1 IgG (M-20, sc-1506, Santa Cruz Biotechnology) (1/50) and a rabbit anti-Gaq IgG (E-17, sc-393, Santa Cruz Biotechnology) (1/25). or ii_b) rabbit anti-Gαq IgG (UCSD, San Diego, California, USA)**(18)** (1/25) and DAPI (1/1000) (D-3571, Molecular Probes); iii) donkey anti-goat IgG (Alexa 488, Molecular Probe) and donkey anti-rabbit (Alexa 568, Molecular Probe) (respectively). Slides were mounted with anti-fade reagent (Molecular Probes).

For confocal laser scanning microscopy, mice were perfused with ice-cold 4% paraformaldehyde for 45min. Once fixed, aortas were carefully dissected and isolated. Permeabilization and all following staining steps were performed in Eppendorf vessels and were similar to those described above for HUVECs. Vessels were open and mounted in anti-fade reagent on glass slides, the internal side of the aorta up. All samples were observed under a BioRad MR600 confocal fluorescence microscope.

PECAM-1 knockout mice

Female PECAM knockout mice and corresponding wide-type control mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). The PECAM strain name is B6129S-Pecam^{tm1Lex} (stock number: 003702), and the wild-type control is B6129SF2/J (stock number: 101045). Mice were used at 2 months of age.

Preparation of Cellular Lysates.

HUVECs were extensively washed with PBS, harvested in lysis buffer (20 mmol/L Tris-HCl pH-7.5, 5 mmol/L EGTA, 150 mmol/L NaCl, 20 mmol/L

glycerophosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 0.1% Tween-20, 1 µg/ml aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L N-tosyl-l-phenylalanine chloromethyl ketone (TPCK), 0.5 mmol/L N(a)-p-tosyl-L-lysine chloromethyl ketone (TLCK)). Extracts were incubated in lysis buffer on ice and then centrifuged (12 000 g, 15 minutes, 4°C). The detergent-soluble supernatant fractions were retained. Protein concentrations in samples were equalized using bicinchoninic acid (BCA) protein assay (BioRad).

Co-Immunoprecipitation

Aliquots of cell lysates (containing 500µg proteins) were pre-cleared for 1.5h at 4°C with 20µl of Protein A/G-Agarose beads (Santa Cruz Biotechnology). Supernatants were incubated overnight with goat anti-PECAM-1 (M-20, sc-1506, Santa Cruz Biotechnology), rabbit anti-Gq (E-17, sc-393, Santa Cruz Biotechnology), or mouse anti-p-Tyr (PY99, sc-7020 Santa Cruz Biotechnology). Antibody concentrations were 8, 4, and 8 µg/ml (respectively). Protein A/G-Agarose beads were then added for a further 1.5h incubation at 4°C. Bound immune complexes were washed 3 times with lysate buffer. Immunoprecipitated proteins were eluted by boiling for 5min in Laemmli sample buffer.

Immunoblot

Denatured immunoprecipitated proteins were separated on 7.5% SDS-polyacrylamide gels and transferred to a PVDF membrane (BioRad). Membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% (v/v) Tween 20 (TBST), membranes were incubated with primary antibodies anti-Gq (E-17, sc-393, Santa Cruz Biotechnology), or anti-PECAM-1 (M-20, sc-1506, Santa Cruz Biotechnology) for 1h in TBST-5%. For the immunodetection of tyrosine-

phosphorylated PECAM-1, membranes were additionally probed with rabbit anti-p-Tyr (P11230 Transduction Laboratories) as a positive control. The bound primary antibodies were detected by using horseradish peroxidase-conjugated secondary antibodies: a goat-anti-rabbit IgG (Pierce) (1/5000) and a rabbit anti-goat IgG (1/2500) (respectively). Enhanced chemiluminescent SuperSignal substrate (Pierce) was used for band visualization. Band intensity (mean optical density integrated for the band area) was quantified on unsaturated x-ray film by a digital image analyser (Bio-Rad Laboratories). All comparisons were made relative to control conditions: sham controls with no shear stress.

Data analysis and statistics.

All experimental values are given as mean and standard error of the mean (SEM). All reported values of n refer number of separate and independent experiments from multiple primary HUVEC cultures. Significant differences between means were calculated with a Student's t test. The Wilcoxon test was used to test for a significant departure of the median from sham control. Statistical significance was taken at the $P<0.05$ level.

RESULTS

Gαq localization in endothelial cells from mice aorta and HUVEC.

To determine Gαq and PECAM-1 localization within endothelial cells *in vivo*, a confocal double-label immunofluorescence study was performed in freshly isolated aortas from wild-type and PECAM-1 knockout mice. In wild-type mice, PECAM-1 was localized along the cell periphery **(Figure 2A)**. No labeling for anti-PECAM-1 was detected in knockout mice **(Figure 2B)**. Similarly, staining with Anti-Gαq also revealed strong Gαq staining along the cell periphery in wild-type mice **(Figure 2C)**. In knockout mice, Gαq was localized near the cell nucleus **(Figure 2D)**. No fluorescence was detected in negative control vessels in which normal serum was substituted for the primary antibody or in vessels incubated with secondary antibody alone (data not shown).

To further investigate the co-localization of Gαq and PECAM-1 in response to specific fluid shear stress profiles, similar experiments were also performed on cultured HUVEC. Both PECAM-1 and Gαq staining was localized to the cell periphery in static HUVEC preparations **(Figure 3A,B)**. Independent of flow profile **(Figure 1)**, PECAM-1 remained localized to the cell periphery **(Figure 3C,E)**. In contrast, cellular localization of Gαq was dependent on exposure to specific flow profiles. When exposed to temporal gradients, Gαq was primarily re-distributed around the cell nucleus **(Figure 3D)**, but when exposed to transient ramped flow devoid of temporal gradients, Gαq remained localized to the cell periphery **(Figure 3F)**.

Impulse but not ramped-transient flow promotes time dependent dissociation of Gαq-PECAM-1complex .

To demonstrate the shear-induced association of Gαq and PECAM-1, both proteins were immunoprecipitated from lysates of HUVEC sham controls, and cells exposed to impulse flow. When Gαq is immunoprecipitated in lysates from both flow profiles, PECAM-1 was found to co-precipitate in the same complex **(Figure 4. left)**. Similarly, when PECAM-1 is immunoprecipitated in lysates from both flow profiles, Gαq was also co-precipitated in the same complex **(Figure 4. right)**.

The kinetics of shear-induced Gαq and PECAM-1 association was determined at 15s, 30s, or 3 min after exposure to each specific flow profile. A rapid disruption of the Gαq-PECAM-1 complex was observed in HUVEC exposed to impulse flow. Dissociation of the complex was maximal after 15s (0.65 ± 0.059 ratio Gαq/PECAM-1 versus sham control) **(Figure 5A)**. The complex rapidly re-associated, and reached a maximum between 30s (0.75 ± 0.057) and 3min (1.13 ± 0.15 ratio Gq/PECAM-1 versus Sham control). The intensity of the Gαq signal immunoprecipitated with PECAM-1 subsequently returned to the sham level. As shown in **Figure 5B**, ramped-transient flow did not induce the dissociation of the Gαq-PECAM-1 complex at either 15s (1.04 ± 0.08), 30s (1.18 ± 0.16), or 3 min (1.01 ± 0.1 ratio Gq/PECAM-1 versus sham control).

Shear induced PECAM-1 phosphorylation

To investigate PECAM-1 phosphorylation as a possible mechanism of impulse flow induced Gαq-PECAM-1 disassociation, tyrosine phosphoylated PECAM-1 was immunoprecipitated from lysates of HUVEC sham controls and cells 15s after exposure to flow. No significant difference in levels of PECAM-1 phosphorylation

was observed between ramped-transient (0.8 ± 0.02 ratio of total PECAM-1/pPECAM-1), and flow and sham controls (0.72 ±0.017 ratio of total PECAM-1/pPECAM-1) (n = 4). However, PECAM-1 phosphorylation was significantly elevated above sham controls in cells exposed to impulse flow (0.98 ±0.03 ratio of total PECAM-1/pPECAM-1)(n = 4)**(Figure 6)**. Inhibition of protein kinases and tyrosine kinases with H7 and genistein (respectively) completely abolished impulse flow induced Gαq-PECAM-1 disassociation and PECAM-1 phosphorylation **(Figure 5C)**.

DISCUSSION

Steady laminar blood flow within the arterial system is generally considered to be atheroprotective, whereas unsteady recirculating flow marked by large temporal gradients has been suggested to be pro-atherogenic **(3)(19)**. Given the pulsatile nature of the cardiac cycle, flow patterns are comprised of two distinct and superposed components, steady and temporally unsteady. Temporal gradients in shear stress are defined as a rapid increase or decrease of shear stress at the same location over a small period of time (<0.3sec). It is the ability of the endothelium to differentially discriminate between these flow components that strongly suggests the existence of distinct molecular mechanisms of mechaochemical signal transduction **(20)(21)(22)**. The molecular components of this transduction pathway remain largely unclear. Both PECAM-1 **(23)** and G proteins **(13)** have independently been suggested as possible mechano-sensing components of the cell. This is the first study to show a link between PECAM-1 and Gαq in response to fluid flow. Furthermore, the association between PECAM-1 and Gαq was shown to differentially discriminate between specific components of fluid flow.

The key finding of this study was the localization of Gαq to the endothelial cell-cell junction and its shear-regulated association to PECAM-1. The importance of PECAM-1 in the localization of Gαq to the cell-cell junction is best illustrated in **figure 2**. In wild-type mice, Gαq forms a distinct ring around the periphery of the cell. This staining pattern is consistent with PECAM-localization, which in the same vessel, is also localized to the periphery of the cell. In PECAM-1 knock-out mice, Gαq was no longer localized to the periphery of the cell and was distributed around the nucleus. The co-localization of Gαq and PECAM-1 was also seen in primary HUVEC.

Although the mechanism of Gαq localization to the cell periphery is unclear, when either Gαq or PECAM-1 were immunoprecipitated from HUVEC lysates, both proteins were found to co-precipitate. This strongly suggests that Gαq is physically associated to PECAM-1 in a complex at the cell-cell junction. It remains possible that other unidentified protein components form the physical linkage between Gαq and PECAM-1, but the PECAM-1-dependent localization of Gαq to the cell-cell junction positions it within the region of greatest shear-induce mechanical tension **(6)**.

The co-localization of Gαq and PECAM-1 was also found to be sensitive to temporal gradients in shear stress. When temporal gradients were eliminated by slowly ramping the onset of flow, it was found that flow alone could not stimulate Gαq-PECAM-1 disassociation. However, when impulse flow was applied to the HUVEC monolayer, temporal gradients were found to be potent mediators of PECAM-1 and Gαq disassociation. As shown in **figure 5**, the kinetics of shear-induced Gαq and PECAM-1 disassociation and re-association was very rapid. The exact time of reformation of the complex varied somewhat between the time points from experiment to experiment, which can be attributed to differences in the batches of primary HUVEC, but was consistent within duplicate analyses inside a single HUVEC harvest. It should be noted that even15 sec. after exposure to impulse flow, the disassociation of the available Gαq-PECAM-1 complexes was never less than 50% of sham control. This may indicate that the distribution of shear-induced tension is not sufficiently strong at all points within the cell-cell junction to promote the disassociation of all the available complexes. Alternatively, given the apparent kinetic rate of the disassociation and re-association, some of the complexes may have already reformed prior to the 15 sec. time point.

This study clearly demonstrates a shear-regulated association of Gαq and PECAM-1 at the endothelial cell-cell junction. This may represent a missing link between the primary force sensing elements of the cell, and downstream mechanochemical transduction pathway. The cytoskeleton has long been suggested to play a role in the downstream mechnochemical transduction pathway **(24)**, but its possible role as a primary sensor of flow has never been convincingly demonstrated. Pharmacological disruption of the actin cytoskeleton does not inhibit shear-induced endothelial NO release **(24)**. Paradoxically, in studies with knock-out mice, the deletion of vimentin, desmin and dystrophin (specialized cytoskeletal components) significantly reduced (but did not eliminate) shear-induced endothelium-dependent vasodilation **(26)(27)(28).** Taken together, these studies suggest that specific elements of the cytoskeleton may be involved in the downstream mechanochemical transduction pathway, but the cytoskeleton as a whole does not seem to play a role as the primary sensor of flow. To date, of the specific cytoskeletal elements shown to be involved in the force transduction pathway, none have been demonstrated to deferentially discriminate between temporal and steady components of shear stress.

Given that the endothelial membrane is directly exposed to fluid shear stress, the lipid membrane itself is more likely to act as, or harbor, the primary components of the shear sensing mechanism. The activation of membrane bound G proteins has recently been recognized as a mediator of flow-induced endothelial response **(29)(30)(14)(31)**. G proteins are one of the earliest known shear responsive cellular elements. G protein activation occurs within 1 second of flow onset **(13)**. When G proteins are isolated and embedded in artificial phospholipid bi-layers in the absence of cytoskeletal elements, Gαq and Gi respond specifically to temporal gradients of shear stress **(4)**. Membrane localization, rapid activation, and force discrimination

strongly implicate G proteins as a primary sensor of fluid shear stress. The activation of Gαq in response to temporal gradients and its localization to the cell-cell junction where rates of tension are the greatest **(6)**, suggest that tension rate may represent the mechanical stimulant for temporal gradients.

The molecular mechanisms of the shear-induced disassociation of the Gαq-PECAM-1 complex remain unexplored. Fujiwara **(10)** has demonstrated that fluid flow induces the tyrosine phosphorylation of PECAM-1. Consistent with this previous report, tyrosine PECAM-1 phosphorylation was significantly elevated only in cells exposed to impulse flow **(Figure 6)**. Although this study was not focused on the possible mechanism of Gαq-PECAM-1 disassociation, this finding suggests a possible mechanism for the differential shear-induced disassociation of the Gαq-PECAM-1 complex. The phosphorylation of tyrosine residue(s) on PECAM-1 may promote the disassociation of Gαq from the complex. The role of PECAM-1 phosphorylation as a possible mechanism is further supported by the finding that inhibition of non-specific cellular tyrosine kinases completely prevented shear-induced Gαq-PECAM-1 disassociation.

In summary, this is the first study to demonstrate a link between PECAM-1 and membrane associated Gαq. The co-localization of the Gαq-PECAM-1 complex to the endothelial cell-cell junction localizes a known shear responsive element within the region of greatest shear-induce mechanical tension. Temporal gradients in shear stress lead to a rapid disassociation and re-association of the Gαq-PECAM-1 complex, whereas transient fluid flow devoid of temporal gradients does not disrupt the complex. Additionally, shear-induced disassociation of the Gαq-PECAM-1 complex is dependent upon the phosphorylation of PECAM-1. The precise nature of the Gαq-PECAM-1 complex and its role in mechanochemical force transduction

remains unexplored. The present study was designed to identify a previously unreported cell-cell junction complex that may represent a missing link between the primary force sensing elements of the cell, and downstream mechanochemical transduction pathway.

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FIGURE LEGENDES

Figure 1. Fluid flow profiles.

Laminar flow profiles: Impulse flow (acute fluid flow marked by significant temporal gradient). Ramped transient (acute fluid flow devoid of significant temporal gradients). HUVEC were harvested 15s, 30s or 3min after the cessation of flow.

Figure 2. *In face* confocal pictures of PECAM-1 and Gq staining in mice aorta preparations.

Wild-type and PECAM-1 knockout mice aortas were fixed by paraformaldehyde perfusion *in situ,* and dissected. Staining was performed using Goat anti-PECAM-1on knockout (A) and in wild-type mice (B). Rabbit anti-Gq stainings were performed in knockout (C) and in wild type mice (D).

Figure 3. Localization of PECAM-1 and Gq in cultured HUVEC 15s after exposure to flow.

In serum-starved confluent primary HUVECs, PECAM-1 localization was detected in cultures exposed to sham (A), impulse flow (C) and ramped-transient flow (E). In both flow profiles, PECAM-1 was exclusively localized along the cell periphery. Gq localization was detected in matched HUVEC cultures exposed to sham (B), impulse flow (D), and ramped-transient flow (F). Gq was primarily re-distributed around the cell nucleus only when exposed to impulse flow.

Figure 4. Typical Gαq and PECAM-1 co-immunoprecipitate in HUVECs exposed to impulse flow.

Gαq and PECAM-1 was immunoprecipitated from HUVECs lysates using polyclonal antibodies. Samples were prepared for Western blot analysis. PECAM-1 co-precipitates with Gαq under both conditions for immunoprecipitation.

Figure 5. Time dependent ratio of Gαq / PECAM-1 immuno co-precipitaion in response to impulse and ramped-transient flow profiles.

Confluent serum-starved HUVECs were subjected to a single impulse of flow (temporal gradient), or to transient ramped flow (devoid of temporal gradients). Cells were harvested 15s, 30s, or 3 min after exposure to flow. Cell lysates were prepared for PECAM-1 immunoprecipitation and Western blot analysis. **A)** Densitometric analysis of nine independent experiments displays the kinetics of association-dissociation of the PECAM-1-Gαq complex following the impulse. Gαq results were normalized against PECAM-1 results obtained from a second immunostaining performed on the same blot. Significant dissociation of the PECAM-1-Gαq complex was maximal after 15s. Association was totally restored at 3min. **B)** Densitometric analysis of seven independent experiments displays the kinetics of association-dissociation of the PECAM-1-Gαq complex following the ramped-transient flow. Gαq results were normalized against PECAM-1 results obtained from a second immunostaining performed on the same blot. PECAM-1-Gαq association did not significantly differ from sham control at any time point. Results are expressed as percentage of sham control (cells not subjected to the impulse). Values are means (±SEM.).(* = $p<0.05$). **C_1)** Disassociation of the Gαq-PECAM-1 complex at 15sec.

C_2) Inhibition of protein kinases and tyrosine kinases with H7 and genistein (respectively) completely eliminated impulse flow induced Gαq-PECAM-*1* disassociation at 15 sec.

Figure 6. Shear induced PECAM-1 phosphorylation in cultured HUVEC 15s after exposure to flow.

Confluent serum-starved HUVECs and cultures additionally treated with H7 and genistein (wide spectrum kinase inhibitors) were subjected to a single impulse of flow (temporal gradient), or to transient ramped flow (devoid of temporal gradients). Cells were harvested 15s after exposure to flow. Cell lysates were prepared for phospho-tyrosine immunoprecipitation and Western blot analysis. No significant difference in levels of PECAM-1 phosphorylation as observed between ramped-transient flow and sham controls. PECAM-1 phosphorylation was significantly elevated above sham controls in cells exposed to impulse flow. (n = 4)(±SEM)(* = $p<0.05$).

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Figure 1

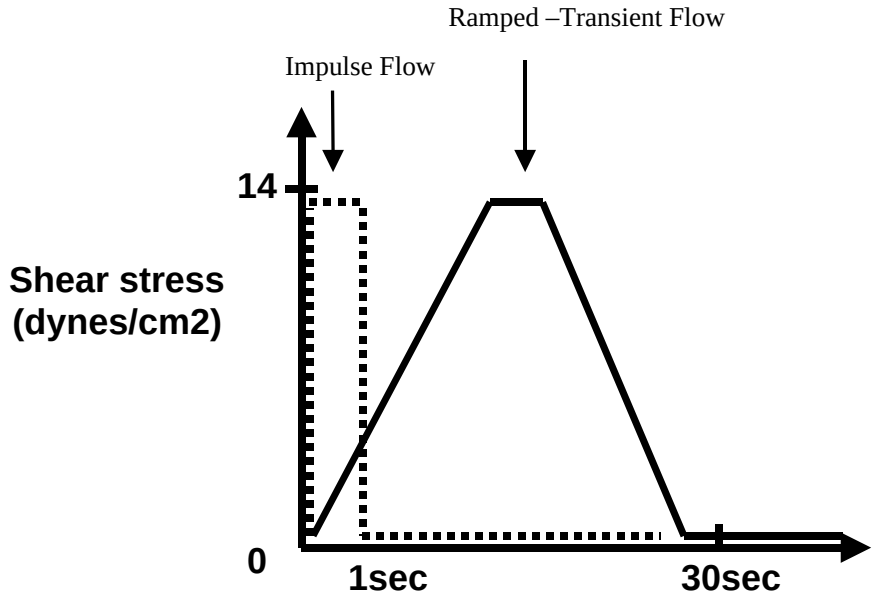

Ramped –Transient Flow
Impulse Flow
14
Shear stress
(dynes/cm2)
0
1sec
30sec

Figure 2

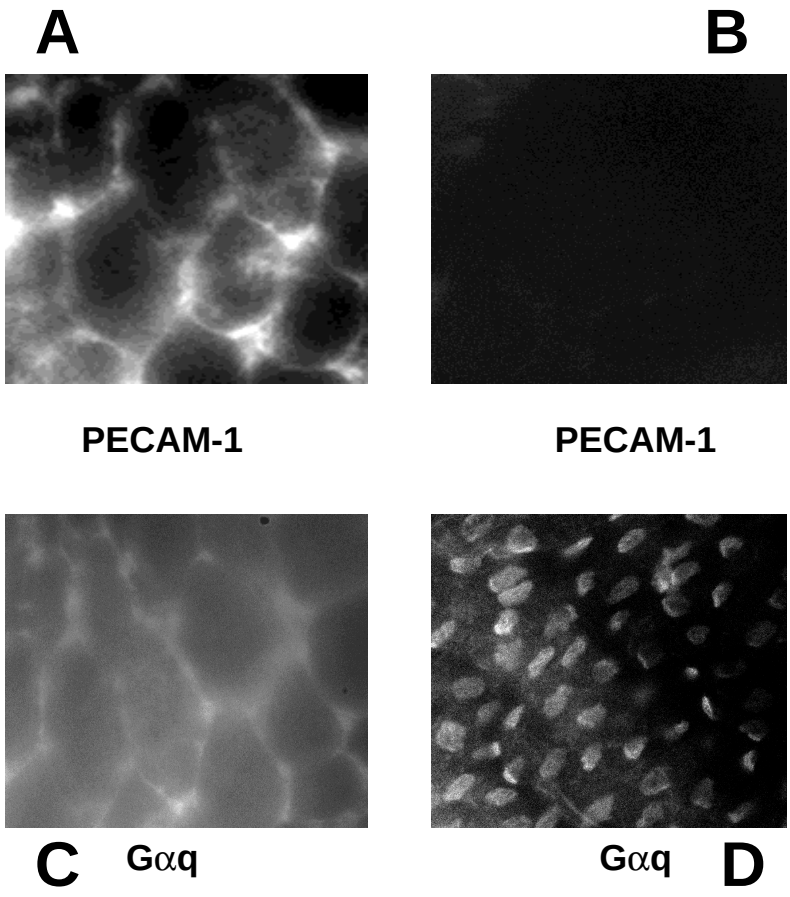


Figure 3

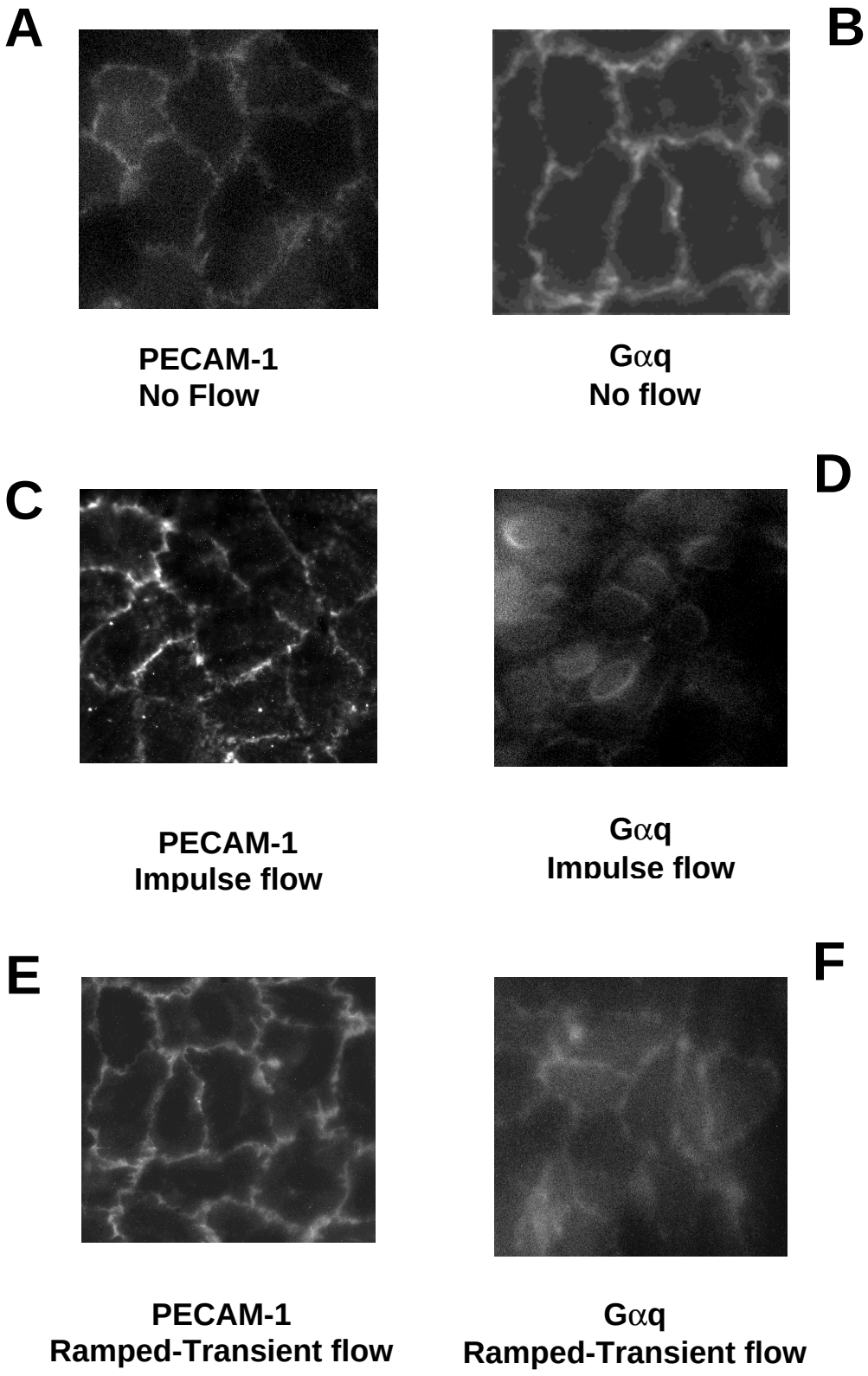

A
B
PECAM-1
No Flow
Gαq
No flow
C
D
PECAM-1
Impulse flow
Gαq
Impulse flow
E
F
PECAM-1
Ramped-Transient flow
Gαq
Ramped-Transient flow

Figure 4

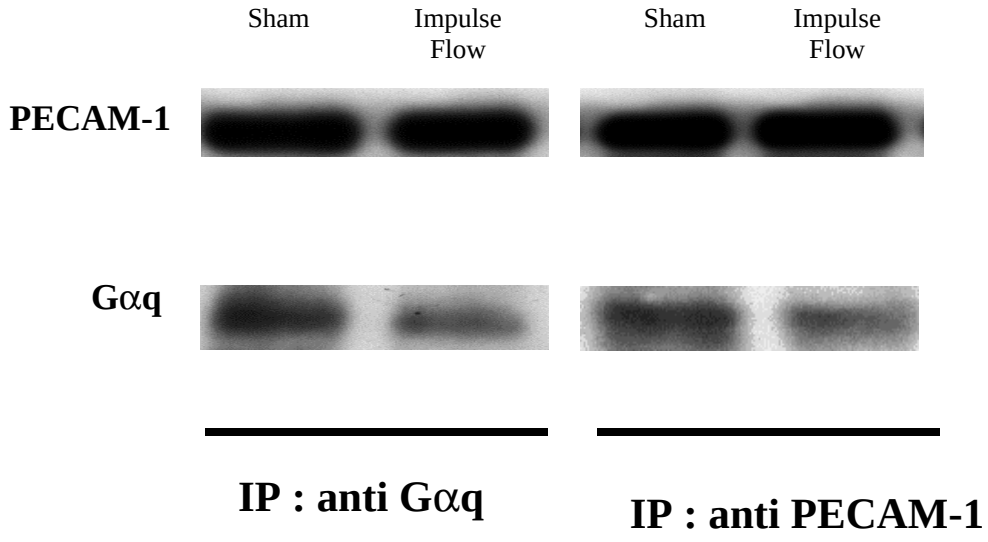

Sham
Impulse Flow
Sham
Impulse Flow
PECAM-1
Gαq
IP : anti Gαq
IP : anti PECAM-1

Figure 5

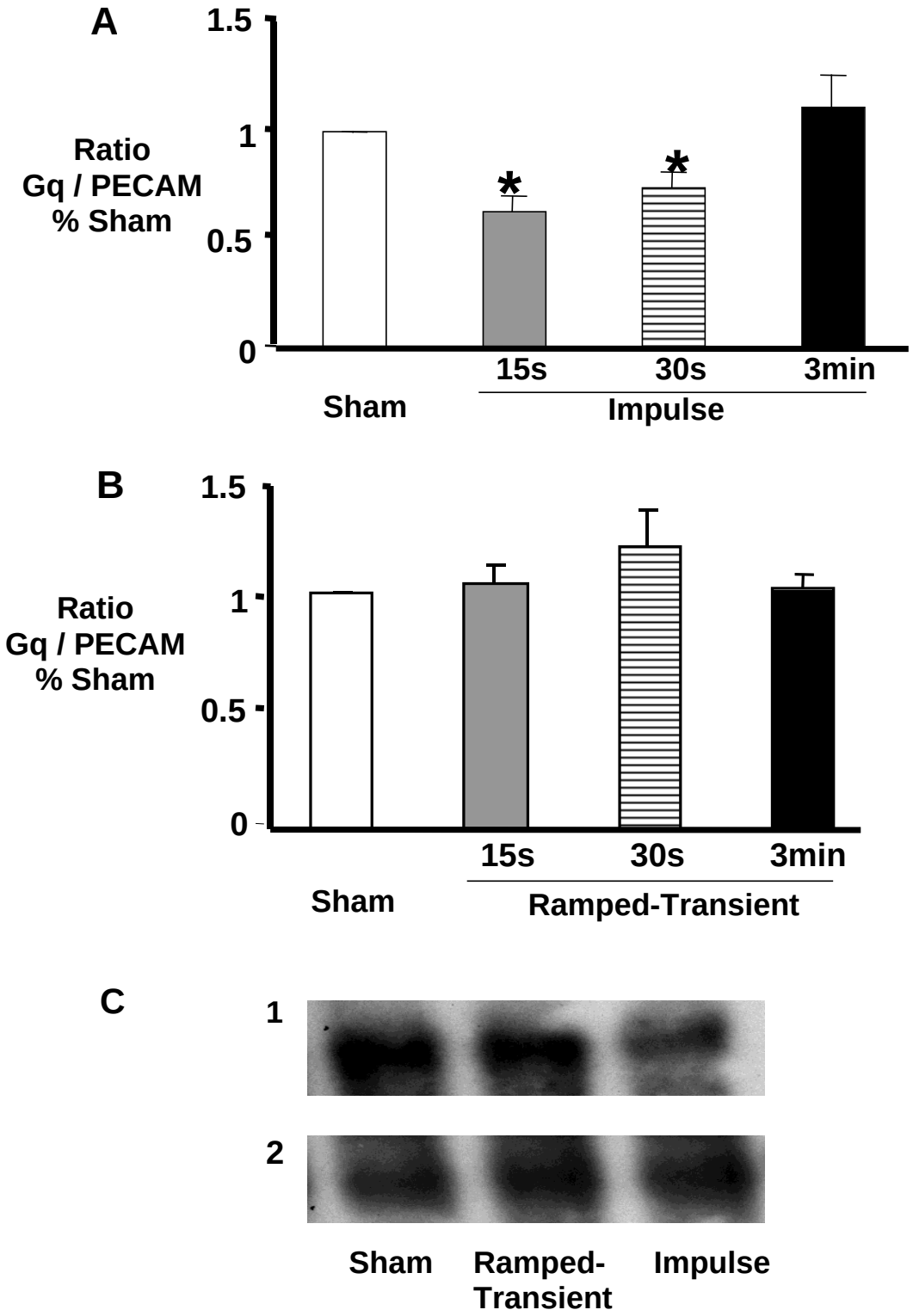
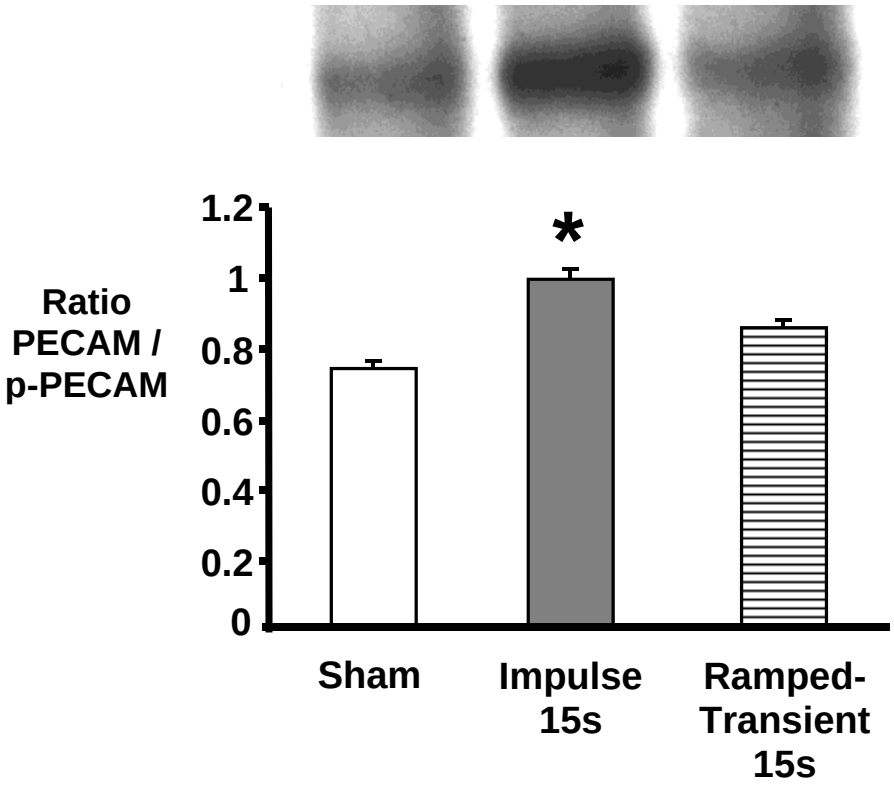

A
1.5
1
0.5
0
Ratio
Gq / PECAM
% Sham
*
*
15s
30s
3min
Sham
Impulse
B
1.5
1
0.5
0
Ratio
Gq / PECAM
% Sham
15s
30s
3min
Sham
Ramped-Transient
C
1
2
Sham
Ramped-
Transient
Impulse

Figure 6


Ratio
PECAM /
p-PECAM
1.2
1
0.8
0.6
0.4
0.2
0
*
Sham
Impulse
15s
Ramped-
Transient
15s