Flow-Related Responses of Intracellular Inositol Phosphate Levels in Cultured Aortic Endothelial Cells

A.R.S. Prasad, Stephen A. Logan, Robert M. Nerem, Colin J. Schwartz, and Eugene A. Sprague

In vitro and in vivo evidence indicates that hemodynamic wall shear stress evokes a diversity of biological responses in vascular endothelial cells, ranging from cell shape changes to alterations in low density lipoprotein receptor expression. The signal transduction mechanisms by which the level of fluid mechanical shear stress is recognized by the endothelial cell and translated into these diverse biological responses remain to be elucidated. The present study focuses on the association between the onset of elevated shear stress and activation of the phosphoinositide signal transduction pathway, as measured by the intracellular release of inositol phosphates, in cultured bovine aortic endothelial cells (BAECs). BAECs were seeded, grown to confluence on large polyester sheets, and preincubated with 0.3 μCi/ml [3H]inositol for 24 hours before insertion in parallel-plate flow chambers for exposure to high shear stress (HS) at 30 dynes/cm² or low shear stress (LS) at <0.5 dyne/cm² for periods ranging from 15 seconds to 24 hours. The induction of HS was associated with an early, transient but significant increase (12%, HS/LS x 100%) in inositol trisphosphate (IP₃) measured at 15 seconds of shear stress exposure followed by a major peak in IP₃ (189%) observed at 5 minutes after HS onset. After these initial increases, IP₃ levels remained double the resting levels within 30 minutes of continued HS exposure and then declined to significantly lower (75%) levels relative to LS-treated cells within 4 hours and remained lower throughout the remainder of the 24-hour HS exposure. LS-treated cells exhibited no significant changes in inositol phosphate levels throughout the 24-hour exposure periods. Exposure of BAECs to shear stress of 60 dynes/cm² resulted in an approximately fourfold increase in IP₃ levels (396%) measured at 5 minutes, almost double the levels measured in cells exposed to 30 dynes/cm² for 5 minutes. Pretreatment of BAECs for 30 minutes with 5 mM neomycin, an inhibitor of phosphoinositide metabolism, before HS exposure inhibited both the early increases in inositol phosphates and subsequent cell elongation and alignment observed in untreated BAECs simultaneously exposed to HS without inhibiting protein synthesis. These results indicate that the exposure of cultured BAECs to elevated wall shear stress is associated with an early biphasic IP₃ increase followed by a resetting of intracellular inositol phosphate concentrations to levels below that observed in static cultured BAECs. Furthermore, neomycin inhibition of this IP₃ response to shear stress is associated with an inhibition of one of the major endothelial biological responses to shear stress, i.e., cell shape change and orientation. These results provide evidence that the phosphoinositide signal transduction pathway may be an important component in transduction of the mechanical shear stress signal into at least some of the vascular endothelial responses to flow. (Circulation Research 1993;72:827–836)

Key words • hemodynamics • shear stress • endothelial cells • inositol phosphates • signal transduction

Hemodynamic forces, including pressure and shear stress, are among the important environmental factors capable of modulating the physiology and pathobiology of the vascular wall. Lining the entire cardiovascular system, the endothelium is positioned to serve as both a sensor of the hemodynamic environment and a transducer of its influence to the underlying vascular cells via growth factors, cytokines, or even direct cell-to-cell communications.¹

Studies performed both in vivo and in vitro have demonstrated that the level of shear stress has an impact on both the physical and functional properties of vascular endothelium. One of the most obvious influences of shear forces is reflected in the endothelial cell shape and alignment patterns observed in vivo in large arteries such as the aorta. Endothelial cells lining the arterial wall in areas exposed to high laminar shear stress (HS) at >10 dynes/cm² exhibit a characteristic elongated shape aligned with the blood flow direction, whereas cells in areas chronically exposed to low shear stress (LS) typically exhibit a polygonal shape without...
any apparent alignment. Recent in vivo studies have demonstrated that this alignment of cells subjected to HS is associated with a redistribution of F-actin from a dense peripheral band, observed in cells residing in LS areas, to a coalescence into large stress fibers crossing cells in a direction parallel to flow.

The responses of endothelial cells to shear stress in terms of geometry, alignment, and cytoskeletal distribution are further reflected in the altered physiology of these cells. On the basis of cell shape and analyses of arterial flow patterns, arterial areas chronically exposed to LS appear to coincide with areas stained with Evans blue in vivo. These Evans blue-stained areas characteristically exhibit an increased cell turnover as well as enhanced influx and accumulation of macromolecules, including fibrinogen, albumin, and lipoproteins.

To establish better the relation between shear stress and endothelial biological responses, a variety of viscometric devices have been developed to model the influence of shear forces on endothelial cells in vitro. Using either cone-plate or parallel-plate viscometers, studies have demonstrated that, as in vivo, cultured endothelial cells exposed to elevated shear stress exhibit elongation, alignment, and redistribution of actin stress fibers parallel with the direction of flow. Further, these morphological cell adaptations are associated with an increase in cell mechanical stiffness. Beyond the ability to verify that cell morphological and structural adaptations to shear stress can be modeled in vitro, recent studies have revealed that an elevated shear stress can induce a number of metabolic responses in cultured endothelial cells. Among the endothelial cell functions reported to respond to elevated shear stress levels are an increased low density lipoprotein (LDL) endocytosis related to an enhanced number of cell surface LDL receptors, an increased synthesis and secretion of prostacyclin and tissue plasminogen activator, and a decreased adherence of platelets and monocytes to the endothelial surface.

Although the cumulated evidence documents that shear stress levels significantly influence endothelial biology, the mechanism(s) by which this mechanical force translates into biological responses remains to be elucidated. Although a number of signal transduction and second-messenger systems are known and may be involved, the present study focuses on the possible role of the plasma membrane–associated phosphoinositide metabolic pathway, emphasizing the adaptation of endothelium to its flow environment. In a previous preliminary report, we provided the first evidence that bovine aortic endothelial cells (BAECs) exposed to HS (30 dynes/cm²) exhibited a peak in intracellular inositol trisphosphate (IP₃) levels at 5 minutes after shear stress initiation followed by a sustained decrease in IP₃ relative to resting levels after a 4–24-hour HS exposure. A recent study examining the response of human umbilical vein cells to elevated shear stress also reported an early increase in IP₃ levels but indicated a major peak at 1 minute. The present study more thoroughly defines the acute response, presents the first data detailing the chronic response of BAECs to shear stress, and examines some possible reasons for differences between the two studies observed in early IP₃ responses. Furthermore, we present the first evidence indicating a possible link between the IP₃ responses and a shear stress–associated biological response, namely, cell shape change and alignment.

Materials and Methods

Bovine aortas collected at a local slaughterhouse served as the source of BAECs. BAECs were isolated enzymatically from aortas as described by Schwartz and were cultured and passaged as reported previously. Briefly, the aortas were rinsed with antibiotics (360 units/ml penicillin and 360 µg/ml streptomycin) containing Dulbecco’s phosphate-buffered saline and then filled to slight distension with collagenase (1 mg/ml, type I, Worthington, Bedford, Mass.) for 15 minutes at 37°C. The endothelial cells that were collected by enzymatic release plus a subsequent rinse of the aortas with Dulbecco’s modified Eagle’s medium (Hazelton Biologics, Inc., Lenexa, Kan.) containing 10% bovine calf serum supplemented with iron (BCSS, Hyclone Laboratories, Inc., Logan, Utah) were centrifuged, resuspended in the same medium, and seeded at density of 10⁴ cells/cm² in 75-cm² culture flasks (Corning Glass Inc., Corning, N.Y.). BAECs were routinely passaged on attaining confluence using 0.25% trypsin. For the shear stress studies, BAECs between passages 2 and 20 were seeded on 10×13-cm 250-µm-thick polyester film sheets (Mylar, Dupont, Inc., Wilmington, Del.) at a density of 2×10⁴ cells/cm² and used within 1 day of attaining confluence. To optimize labeling of cellular phosphoinositides, BAECs were preincubated in an inositol-free Dulbecco’s modified Eagle’s medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 0.3 µCi/ml [³H]inositol and 10% BCSS 24 hours before exposure to shear stress. This medium was also used as the circulating medium throughout shear stress exposure. Verification of endothelial cell culture purity was ascertained by growth patterns and cell morphology as well as by the presence of factor VIII antigen.

Shear Stress Studies

To provide the large number of cells requisite for the phosphoinositide studies, parallel-plate flow chambers previously used in this laboratory were redesigned to provide a 90-cm² cell surface area for shear stress exposure. Each chamber consisted of two horizontal parallel glass plates separated by a spacer/gasket of either 200-µm (HS) or 2.0-mm (LS) thickness, creating a long rectangular flow channel (7×14 cm) whose height was much less than either its length or width. The top and bottom plates were housed within molded nylon units, the top half of which contained inlet and outlet flow connections. Laminar flow was established across the width of the channel via a reservoir at either end of the channel formed by narrow slots in the top plate that were cut perpendicular to the direction of flow and placed immediately beneath the flow connectors. The entire apparatus was contained within an aluminum frame with attached clamps to permit sealing of the chambers. Confluent [³H]inositol-prelabeled BAECs residing on the large polyester sheets described above were inserted upon the bottom glass plate of the two parallel plates. The wall shear stress experienced by cells in these chambers was directly related to the flow rate of the circulating medium through the channel and inversely to the square of the height of the channel.

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Flow rate was gravity driven and governed by the height of separation between the upper and lower media reservoirs using a peristaltic pump (Cole-Parmer Instrument Co., Chicago, Ill.) to return the medium to the upper reservoir. Flow rate was continuously monitored by an in-line flowmeter (Cole-Parmer). By use of this flow system at a flow rate of 1.25 ml/sec, a wall shear stress of 30 dynes/cm² can be generated with a Reynold’s number of 30 at a channel height of 200 μm. At such a Reynold’s number, the flow is laminar, and the velocity profile is that of a fully parabolic form over more than 98% of the channel length. The same channel with the 2-mm spacer yields a wall shear stress of approximately 0.5 dynes/cm² at the same laminar flow rate. To attain shear stress rates of 60 dynes/cm², the flow rate was doubled while maintaining the 200-μm channel height. The construction design of these chambers also permits continuous microscopic visualization of cells residing within the flow channel during shear stress regimens.

Each chamber (HS and LS) was positioned in a separate flow circuit. The temperature of the circulating [3H]inositol containing the medium was maintained at 37°C, and the pH was held constant at 7.4 by maintaining an atmosphere of 5% CO₂ in the reservoirs. By use of this experimental apparatus, BAECs were exposed to either HS or LS for times ranging from 30 seconds to 24 hours. The designated stress exposure time was terminated by perfusion with and fixation of the cells in situ under flow using ice-cold 5% trichloroacetic acid (TCA) introduced by opening a three-way valve to a TCA-containing reservoir positioned at the same height as the upper reservoirs. As soon as the BAECs were covered by TCA, the flow was stopped, and the sheets bearing the fixed cells were removed from the flow chambers for extraction and analysis of inositol phosphates as described below.

Inositol Phosphate Analyses

Inositol phosphate analyses were performed according to the method of Berridge et al. ²⁸ Specifically, the sheets bearing the in situ fixed BAECs were transferred to dishes containing cold 5% TCA for 30 minutes to allow extraction of the water-soluble inositol phosphates. After removing and combining this extract with two subsequent water rinses, the extract was washed four times with ethyl ether and neutralized with 6.25 mM sodium tetraborate.

After extraction, the inositol phosphates were isolated by anion exchange chromatography using 10-m1 columns containing Dowex AG 1-X8 (100–200 mesh, formate form, Bio-Rad Laboratories, Richmond, Calif.). Columns were washed with 60 mM ammonium formate in 5 mM sodium tetraborate, and the [3H]inositol monophosphates ([3H]IPs), [3H]inositol bisphosphates ([3H]IP₂s), and [3H]IP₂s were eluted sequentially with 0.2, 0.4, and 1.0 M ammonium formate in 0.1 M formic acid, respectively. Samples (1 ml) were collected in scintillation vials containing 10 ml Hydrofluor (Beckman) cocktail and counted in a Beckman model LS 5801 liquid scintillation radiometer. A total of 5 ml was collected for each inositol phosphate. All inositol phosphate levels were normalized to total cellular DNA concentrations. Because of variability in absolute levels of inositol phosphate per milligram DNA between experiments, results were converted to HS/LS ratios to allow group comparisons.

For phosphoinositide analyses, the cell residue remaining after TCA extraction was scraped from the sheets with methanol and extracted with chloroform: methanol (1:2 [vol/vol]). The chloroform phase was removed and concentrated, and the extracted phospholipids were fractionated on silica gel H thin-layer chromatography plates. The individual phospholipids were localized by viewing the plates under ultraviolet light after spraying with rhodamine 6G. The areas corresponding to the individual phosphoinositides as identified by standards were scraped, and the phospholipids were digested with HClO₄. After addition of water and centrifugation, aliquots of the supernatant were removed for counting radioactivity and for determination of phosphorus (P) for normalization of results. P was quantitated by the method of Bartlett. ²⁹

To ensure that the changes in [3H]inositol phosphate levels measured did not simply reflect a change in the specific activity of the cellular inositol pool, mass measurements of IP₃ were performed in selected studies using a commercially available radioimmunoassay (Amersham Corp., Arlington Heights, III.).

Statistical Analyses

The mean concentrations of intracellular inositol phosphates measured within the respective shear stress treatment groups (HS relative to LS) were compared by Student’s paired t test.

Results

These studies were designed to test the hypothesis that the initiation of elevated shear stress triggers an early increase in cellular inositol phosphate levels similar to that observed in phosphoinositide-linked receptor systems on ligand–receptor interaction.²⁰ To address this issue, cells in parallel circulation systems were exposed to either HS (30 dynes/cm²) or LS (0.5 dyne/cm²) for periods of 30 seconds to 5 minutes at completion of shear exposure time; the cells were immediately fixed by TCA in situ while maintaining flow conditions to prevent metabolic changes in relative inositol phosphate concentrations after the cessation of flow before fixation. Analysis of the initial TCA fixation fluid indicated that measurable amounts of [3H]-labeled inositol phosphates were not extracted during this brief fixation. As illustrated in Figure 1, a significant sharp peak in intracellular levels of IP₃ (189.4±27.1%, p<0.001) as well as IP₂ (190.8±31.1%, p<0.001) and IP (144±20%, p<0.001) was observed in BAECs exposed to HS (30 dynes/cm²) for 5 minutes, which returned to baseline levels (LS) within 30 minutes (see Figure 4).

In addition to this major peak in inositol phosphates observed at 5 minutes, an earlier, transient, and lower peak in endothelial IP₃ levels was observed between 30 seconds and 2 minutes, although the increases were only significantly higher than paired LS-exposed BAECs at 30 seconds (130±8%, p<0.05) and 1 minute (143±27.1%, p<0.01). IP levels also exhibited an early significant increase (137±17%, p<0.05) at 1 minute after the onset of HS. Although no significant increase in IP₃ levels was observed within this time frame, these early increases in both IP₂ and IP suggested that there may have been an early transient IP₃ response before the initial 30-second
levels phosphate for 30 seconds Error...graph showing response of intracellular levels of inositol phosphates within the initial 5 minutes of bovine aortic endothelial cell (BAEC) exposure to elevated shear stress (30 dynes/cm²). IP₁, inositol monophosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate; HS, high shear stress; LS, low shear stress. Confluent BAECs cultured on solid polyester film substrate were preincubated for 24 hours in 0.3 μCi/ml [³H]inositol in inositol-deficient Dulbecco’s modified Eagle’s medium—10% bovine calf serum supplemented with iron before insertion into large parallel-plate flow channels. Maintaining the preincubation medium as the circulation medium, BAECs were exposed to either LS (<1 dyne/cm²) or HS (30 dynes/cm²) for 30 seconds to 5 minutes. A separate set (n=3) of experiments was performed at 15 seconds of HS exposure for analysis of IP₁ only (measured by radioimmunoassay), and the results are presented as a separate point connected to other data by a dashed line. On completing the time exposure, cells were fixed in situ under flow with trichloroacetic acid and removed, and the inositol phosphates were extracted and measured as described in “Materials and Methods.” Inositol phosphate levels in cells exposed to HS were first normalized to total cellular DNA and then expressed as a percentage of levels measured in LS-treated BAECs in paired experiments (n=7). Basal inositol phosphate levels measured ranged from 8.27 to 21.44 disintegrations per minute (dpm)/μg DNA for IP₁, from 1.01 to 2.47 dpm/μg DNA for IP₂, and from 0.97 to 2.22 dpm/μg DNA for IP₃. Error bars represent SEM.

To ensure that the peak response in [³H]IP₂ levels at 5 minutes did not simply reflect a change in the specific activity of the inositol pool, IP₁ levels were also measured using the RIA method specific for inositol 1,4,5-trisphosphate. Similar to results obtained using chromatographic separation of [³H]IP₁, RIA measurements of IP₁ in BAECs at 5 minutes of HS exposure indicated a 208±23.0% (p<0.001) increase relative to 5-minute levels in BAECs exposed to LS or no shear stress. In addition to the IP₁ measurements, phosphoinositide diphosphate (PIP₂) levels were measured in cells exposed to either LS or HS for 5 minutes in a total of four experiments to provide evidence that changes in IP₁ were mediated via phospholipase C-mediated PIP₂ hydrolysis. Although the absolute levels of PIP₂ varied greatly among individual experiments, the relative ratio between HS- and LS-treated BAECs was relatively constant at a mean of 59.8±3.4% (HS/LS×100%, SEM, p<0.01), consistent with utilization of PIP₂ to generate IP₃.

To compare shear stress–related intracellular generation of inositol phosphates with that observed in response to a known receptor-linked IP₃ activation, confluent BAEC cultures were challenged with a dose of bradykinin (final concentration, 1 μM) previously shown to elicit a maximal response of IP₃ generation in BAECs. As can be seen in Figure 2, bradykinin stimulation elicited an IP₃ response of 454% within 15 seconds after initiation of stimulation. Note that after 15 seconds the IP₃ levels rapidly decreased while the peak responses of the IP₃ metabolites, IP₂ and IP₁, were successively delayed. Thus, the peak IP₃ response to bradykinin is not observed until 1 minute after stimulation.

The contrast between the maximal IP₃ response to bradykinin (454%) relative to the shear stress stimulus (189%) raised the question of whether this magnitude of the IP₃ response to shear stress could be further increased in response to an increase in shear stress levels above 30 dynes/cm². As illustrated in Figure 3, increasing BAEC shear stress exposure to 60 dynes/cm² was associated with a 396±24.4% increase in IP₃ levels measured at 5 minutes relative to LS-treated BAECs, whereas BAECs exposed to 30 dynes/cm² exhibited a 196±15.7% increase, similar to that observed in the initial study in this separate set (n=3) of experiments. Thus, a twofold increase in shear stress level resulted in an approximate doubling of the intracellular IP₃ response.

Although most studies examining cellular IP₃ levels as a second-messenger signaling mechanism have focused on immediate or short-term responses to receptor activation, these studies were also designed to examine...
Inositol phosphate responses to shear stress exposure. This approach is based on some of the biological adaptations of endothelial cells to shear stress, such as cell shape,9 decreases in cell proliferation rate,21 and increases in LDL receptor,11 which all require relatively long (hours) shear stress exposure for expression. In contrast to the early peak in intracellular inositol phosphate levels observed at 5 minutes after induction of elevated shear stress, Figure 4 illustrates that BAECs exposed to long-term elevated shear exhibited a return from the peak response observed at 5 minutes to initial preexposure IP$_3$ levels within 30 minutes, followed by a continuing statistically significant decline to lower levels ($p<0.001$) at 4 and 24 hours. Intracellular IP$_3$ levels attained a mean level of 59% (4 hours) and 72% (24 hours), relative to low shear cell levels measured at 4 and 24 hours, respectively. Although both of these levels were significantly lower than their respective LS control levels, they were not significantly different from each other. The data presented in Figure 4 are composites of several individual paired (LS and HS) experiments. Each experiment included a paired set of cells exposed to LS and HS for 5 minutes plus two paired sets exposed for longer shear stress periods. Note data from one typical experiment presented in Table 1. To determine whether this apparent decrease in IP$_3$ levels after 24 hours might be related to a change in the specific activity within the intracellular inositol pool, IP$_3$ levels at 24 hours were also measured using RIA in a separate set ($n=4$) of experiments. By use of this more direct measurement of IP$_3$, significantly lower levels of IP$_3$ (79.8±5.3, $p<0.01$) were again measured in BAECs preconditioned to 24-hour HS relative to cells exposed to LS for the same period. The absolute RIA values of IP$_3$ measured in one of the composite experiments are detailed in Table 1. Note that, although the IP$_3$ concentrations of LS-treated BAECs measured in this paired experiment did not change across the 24-hour period, IP$_3$ levels for HS-treated cells more than doubled at 5 minutes and decreased to much lower levels (70%) by 24 hours. In an effort to confirm that IP$_3$ levels for LS-exposed cells did not exhibit any significant increase or decrease across long-term studies, a series of more extensive studies involving only LS exposure of BAECs was performed. This allowed us to perform all
ments in our laboratory have demonstrated that pre-treatment of BAECs for 30 minutes with 5 mM neomycin sulfate blocked 90–100% of the IP₃ response to either bradykinin or LDL. In a separate set of paired experiments (n=4), BAECs were similarly pre-treated with neomycin (Sigma Chemical Co., St. Louis, Mo.) followed by exposure to HS (30 dynes/cm²) for 5 minutes in the continued presence of neomycin before fixation in situ as above. Simultaneously, parallel HS runs using untreated BAECs were conducted. As depicted in the top panel of Figure 6, the IP₃ response to HS at 5 minutes in the control cells (186.2±20.6% relative to LS control) was almost totally inhibited in BAECs pretreated with neomycin (105.5±8.5% relative to LS). As expected, BAECs pretreated with neomycin and exposed to LS for 5 minutes exhibited lower IP₃ levels (79.4±4.9%) relative to untreated LS cells. Consistent with these findings, BAECs preincubated with neomycin and then conditioned to HS for 24 hours in the continued presence of neomycin exhibited even lower IP₃ levels (44.1±5.2%, n=3) than those levels (71.0±7.1%) detected in untreated HS-conditioned cells, as measured by RIA and expressed as percentage of untreated LS control cell IP₃ levels. Furthermore, when neomycin-pretreated BAECs were subjected to 24-hour HS while maintaining 5 mM neomycin in the circulating medium, cell shape and alignment remained unchanged relative to static cultured BAECs throughout the experiment, whereas untreated cells that were run in parallel exhibited the typical elongation and alignment with flow (Figure 6, middle and bottom panels). Special care was taken to maintain pH at the same level in both treatment and control circulating media. Trypan blue staining and the ability of these cells to survive and proliferate after this prolonged neomycin exposure indicate that cell viability was maintained. To exclude the possibility that neomycin might be exerting its effect on cell alignment via a general inhibition of protein synthesis, two sets (n=4) of BAEC monolayers were preincubated with either control or culture medium containing 5 mM neomycin for 24 hours at 37°C. As a positive control, a third set of BAECs was preincubated with cycloheximide (100 μg/ml) for 30 minutes. After their respective preincubations, the media of all plates were changed to M199 (deficient in cysteine-methionine) containing 1 mg/ml [35S]cysteine-methionine (Amersham), and the BAECs were incubated for an additional 4 hours at 37°C. The cells were then rinsed with phosphate-buffered saline and lysed with 10% sodium dodecyl sulfate. The proteins within the lysates were precipitated with 10% TCA, centrifuged, and redissolved for counting radioactivity. Analysis of the data indicated that the amount of labeled cysteine and methionine incorporated by neomycin-treated cells was not significantly different from cells incubated with control media. On the other hand, BAECs preincubated with cycloheximide for 30 minutes exhibited a 91.4% inhibition in labeled amino acid incorporation into protein.

**Discussion**

The results of this study indicate that the onset of shear stress exposure related to flow across cultured confluent BAECs is followed by a biphasic response in intracellular IP₃ concentrations characterized by a rela-

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**Figure 4.** Graphs showing response patterns of the inositol phosphates to high shear stress (HS) across a 24-hour time period in confluent bovine aortic endothelial cells (BAECs). IP, inositol monophosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate. Cells prelabeled with [3H]inositol were exposed to either HS (30 dynes/cm²) or low shear stress (LS, <1 dyne/cm²) for 24 hours simultaneously in parallel flow systems before fixation in situ with 5% trichloroacetic acid and extraction and analysis of inositol phosphate concentrations as described in "Materials and Methods." Inositol phosphate concentrations in BAECs exposed to HS were first normalized to total cellular DNA and then expressed as a percent relative to paired LS control cells (n=6). Error bars represent SEM.
tively small transient peak within the first 15 seconds, followed by a major peak at 5 minutes after the induction of HS. By 30 minutes, these elevated inositol phosphate levels return to control preshear levels in the presence of continuing exposure to HS. Finally, this early peak in IP₃ levels in cells exposed to an elevated shear stress is followed by a decline to IP₃ levels significantly below both initial resting and LS levels beginning at 2 hours and reaching an apparent low plateau between 4 and 24 hours.

One of the aims of the present study was to determine if an increase in intracellular IP₃ levels analogous to that observed for receptor–ligand interaction might provide a signal transduction pathway linking the shear stress stimulus to the wide variety of shear-associated endothelial cellular responses. A sharp rise in intracellular IP₃ levels is now a well-documented sequela to activation of many cell surface receptors on ligand binding.²⁰ Furthermore, IP₃ acts as a second-messenger molecule, along with diacylglycerol released simultaneously on hydrolysis of phosphoinositide diphosphate, to link receptor activation to cell responses, including releases of intracellular calcium stores, cytoskeletal rearrangements, and secretory responses. Depending on the specific ligand–receptor coupling examined, the shear stress–related pattern of intracellular IP₃ response both contrasts and resembles previously documented IP₃ responses to ligand–receptor coupling. In contrast to the somewhat delayed shear-induced IP₃ response, BAECs treated with bradykinin exhibit peak intracellular IP₃ levels that increase 400% or more relative to resting levels within 15 seconds after treatment.²³ This response pattern is typical of receptor-activated pathways directly coupled to the phosphoinositide signal transduction pathway, leading to a release of endoplasmic reticulum stores of calcium.²⁴ Despite the contrasts of the shear-related IP₃ response pattern with this more “typical” cellular response, it has distinct analogies to the pattern of cellular IP₃ increases observed in response to certain growth factors. Specifically, an IP₃ response pattern typified by an early transient increase (within the first minute) followed by a much larger sustained peak between 5 and 10 minutes has been reported in fibroblasts treated with platelet-derived growth factor²⁴ and basic fibroblast growth factor²⁵ and in tumor cells treated with epidermal growth factor.²⁴

The delayed response of the major IP₃ peak observed on interaction of platelet-derived growth factor, basic fibroblast growth factor, and epidermal growth factor with their respective receptors appears to be related to two primary factors. First, each of these growth factors activates receptors possessing intrinsic tyrosine kinase activity that, in turn, phosphorylate and secondarily activate phospholipase C-γ. Providing additional evidence linking these pathways, recent studies have demonstrated that overexpression of phospholipase C-γ in fibroblasts leads not only to increased phosphorylation of this enzyme in response to platelet-derived growth factor and basic fibroblast growth factor but also to increased phosphoinositide hydrolysis and intracellular IP₃ levels.²⁵,²⁶ Second, evidence is accumulating that the IP₃ comprising the major response peak to these growth factors is actually inositol-1,3,4-trisphosphate, whereas the early transient IP₃ response to platelet-derived growth factor and basic fibroblast growth factor and the sharp early response to bradykinin is the more abundant IP₃ isomer inositol-1,4,5-trisphosphate.²⁷ Activation of platelet-derived growth factor receptors appears, therefore, to activate tyrosine kinase–mediated phosphorylation of phospholipase C-γ and also trigger phosphatidylinositol-3-kinase–mediated phosphorylation of phosphatidylinositol-4-phosphate to provide a novel substrate, phosphatidylinositol 3,4-phosphate, for activated phospholipase C-γ.

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IP₃, inositol trisphosphate; HS, high shear stress; LS, low shear stress. Values are mean±SD of three replicate measurements.

*Measured by radioimmunoassay.

**TABLE 1. Intracellular Inositol Trisphosphate Concentrations for a Representative Experiment of Bovine Aortic Endothelial Cells Exposed to Either Low or High Shear Stress for Increasing Amounts of Time**

![Graph showing intracellular inositol trisphosphate (IP₃) concentrations (disintegrations per minute [DPM] per microgram total cellular DNA) in bovine aortic endothelial cells (BAECs) exposed to low shear stress (0.5 dyne/cm²) for increasing times up to 24 hours. These represent a separate set of experiments designed to examine response of five sets of BAECs to low shear stress across time. Error bars represent SEM.](http://circres.ahajournals.org/)

**FIGURE 5.** Graph showing intracellular inositol trisphosphate (IP₃) concentrations (disintegrations per minute [DPM] per microgram total cellular DNA) in bovine aortic endothelial cells (BAECs) exposed to low shear stress (0.5 dyne/cm²) for increasing times up to 24 hours. These represent a separate set of experiments designed to examine response of five sets of BAECs to low shear stress across time. Error bars represent SEM.
Figure 6. Influence of neomycin on bovine aortic endothelial cell (BAEC) inositol trisphosphate (IP₃) and cell shape and alignment responses. Top panel: Bar graph showing IP₃ concentration plotted against shear level. During the last 30 minutes of the 24-hour [³H]inositol-labeling period, neomycin (Neo) was added at a final concentration of 5 mM to confluent BAECs. After the preincubation period, BAECs were subjected to high shear stress (HS, 30 dynes/cm²) for 5 minutes before fixation in situ and measurement of IP₃ levels as described in "Materials and Methods." Controls consisted of shear stress experiments on untreated BAECs exposed to HS and low shear stress (LS, <1 dynes/cm²) simultaneously with treated BAECs but in parallel flow circuits (n=4). Error bars represent SEM. Middle and bottom panels: Photomicrographs of BAECs. Confluent BAECs exposed to 24 hours of HS (30 dynes/cm²) after a 24-hour preincubation with [³H]inositol and, for the last 30 minutes of preincubation, in the presence (middle panel) or absence (bottom panel) of 5 mM Neo as described above. Neo was maintained in the circulating medium throughout the shear stress exposure. Cells were photographed in situ under flow at the end of the 24-hour shear stress exposure using a Nikon Diaphot photomicroscope. Magnification, ×80.

Thus yielding inositol-1,3,4-trisphosphate. Although the prerequisite for phospholipase C-γ phosphorylation provides one plausible explanation for the delayed IP₃ response to shear stress, the possibility that the major IP₃ peak observed in this study is comprised of primarily inositol-1,3,4-trisphosphate is argued against by the fact that similar results were obtained in our studies using either the chromatographic or the RIA methods. According to RIA distributors (Amersham), inositol-1,3,4-trisphosphate exhibits only a 0.22% cross-reactivity with the antibody to inositol-1,4,5-trisphosphate provided in the assay system.

Another parallel of the shear-induced pattern of intracellular IP₃ release and that associated with platelet-derived growth factor is the temporal relation between IP₃ increases and releases of intracellular calcium stores. With agents such as bradykinin, the early large IP₃ increase directly triggers a secondary release of intracellular calcium. In contrast, several groups have demonstrated that platelet-derived growth factor triggers an early intracellular calcium release that precedes the major increase in IP₃. Similarly, shear stress has been reported to trigger large increases in intracellular calcium in BAECs within the first minute, well before the major IP₃ peak demonstrated in our study. Thus, Geiger et al reported that peak calcium responses in cultured BAECs exposed to HS levels similar to those used in this study (30 dynes/cm²) were observed at 42 seconds. Whether the early transient IP₃ peak we observed at 15 seconds after shear initiation could provide the trigger for this remains unknown. What is apparent is that the major IP₃ peak at 5 minutes does not seem to correlate with any similar later peak in intracellular calcium, which appears to return to baseline within 3
minutes and become relatively refractory to any further stimulation. Thus, it seems possible that the IP$_3$ released at 5 minutes may either be compartmentalized or have another intracellular target or function aside from calcium release.

The patterns of IP$_3$ response to shear observed in this study differ from those presented in a recent study by Nollert et al., in which the authors report a greater than 200% increase in IP$_3$ levels in human umbilical vein endothelial cells within 30 seconds of exposure to an elevated shear stress (22 dynes/cm$^2$). This early peak gradually declined to near 150% of resting cell IP$_3$ concentrations at 6 minutes, followed by a return to control levels after 15 minutes of exposure to elevated shear stress. Among the possible explanations for this difference in IP$_3$ response patterns compared with our results are the obvious differences in species and in tissue source, i.e., venous versus aortic. Furthermore, the shear stress times in our studies are based on the time between shear onset and fixation of the BAECs at the specified in situ shear stress. This is in contrast to the study of Nollert et al, in which the umbilical vein cells were not fixed until after cessation of shear stress and after removal from the shear stress apparatus. Thus, under this latter design, the cells would have experienced both a sudden increase and decrease in shear stress as well as the requisite time for removal before the fixation of the cells required to inactivate phosphoinositide metabolism.

A contrasting feature of the shear stimulus in vivo and as applied in these studies relative to typical ligand–receptor interactions is its continuous nature. Whereas a typical hormone or cytokine interaction with cellular receptors is usually episodic, endothelial cells throughout much of the arterial system are exposed to chronic, though pulsatile, shear forces. A unique aspect of the present study is the measurement of inositol phosphate levels throughout prolonged exposure of BAECs to elevated shear stresses. The discovery that, after the initial peak IP$_3$ response, intracellular IP$_3$ levels actually decline to an apparent new steady state, significantly lower than initial resting levels between 4 and 24 hours, poses the possibility that these lower but much more prolonged levels may also serve as a signal to set and maintain some of the more chronic biological responses of the cell. Alternatively, the long-term IP$_3$ response to shear stress may reflect adaptation of the plasmalemma to the chronic physical environment secondary to other intracellular signals or metabolic changes. If this difference in inositol phosphate levels observed between endothelial cells exposed to long-term HS and LS in vitro is reflective of in vivo differences between LS and HS areas of the arterial bed, then these differences could provide a potential background for a difference in cellular sensitivity and reactivity to agents that activate the phosphoinositide-signaling pathway subsequent to receptor binding in HS relative to LS areas.

Although we have demonstrated a consistent and characteristic response of intracellular inositol phosphate levels in BAECs exposed to the stimulus of an elevated steady-state laminar shear stress, the linkage between the response of these intracellular signaling molecules and the wide array of endothelial biological responses to shear stress has yet to be elucidated. In the present study, we present evidence indicating that an inhibition by neomycin in the generation of inositol phosphates from membrane polyphosphoinositides also inhibits the cell shape change and flow alignment so characteristic of the endothelial cell response to elevated shear stress both in vitro and in vivo. Neomycin has a high affinity for polyphosphoinositides and, thus, potentially blocks IP$_3$ and diacylglycerol generation by making substrate unavailable to phospholipase C. Although these data do not exclude other possible cellular effects of neomycin, our results indicate that cells treated with neomycin remained viable and that exposure of cells to 5 mM neomycin did not inhibit cellular protein synthesis. Further, these results do not distinguish between IP$_3$ and diacylglycerol or both molecules being critical to activation of the cell shape and alignment responses. Whether other biological responses to shear stress are similarly inhibited by neomycin and whether inhibitors or stimulators of specific key molecules in the phosphoinositide activation sequence, including G proteins, will influence specific responses to shear stress pose intriguing questions for further investigation.

It should be emphasized that this study has focused on what we believe to be one of the important second messengers in the transduction of the shear stress signal. Our results provide no specific insight into the recognition event, i.e., the mechanisms involved in an endothelial cell recognizing flow and discriminating between different types of flow environments. As such, we cannot say whether the influence of flow is related directly to the shear stress imposed, to a shear-dependent convection-diffusion-coupled transport mechanism, to some other flow-related factor, such as the velocity profile or particle residence time, or even to all of these. At issue, in this case, is whether shear stress is directly recognized by some mechanosensitive “receptor” or cellular complex such as the cytoskeleton or ion channel or whether a flow-associated factor triggers a change in local concentration of molecules such as ATP at the cell surface, which, in turn, activates phosphoinositide metabolism through known endothelial purinergic receptors. Although the medium used in these studies does not contain ATP, this does not exclude a possible flow-associated release of this molecule from intracellular sites or influence on ectonucleotidases. Therefore, whether or not the phosphoinositide system is directly stimulated by shear stress or whether its induction is due to some other flow-related factor needs further investigation.

In summary, these studies indicate that confluent cultured BAECs exhibit a biphasic response in intracellular inositol phosphate levels when exposed to chronic elevated shear stress for 24 hours. Integration of these data with studies examining other flow-influenced signaling systems, including changes in ion channel function, membrane fluidity, and intracellular calcium, may begin to elucidate the signal transduction pathway between the mechanical stimulus of elevated shear stress and a variety of cellular biological responses.
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