Vol. 170, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1990 Pages 281-287

SHEAR STRESS INCREASES INOSITOL TRISPHOSPHATE LEVELS IN HUMAN ENDOTHELIAL CELLS

M. U. Nollert[1], S.G. Eskin[2], and L.V. McIntire[1,*]

1 Biomedical Engineering Laboratory, Rice University, Houston,TX 77251

2 Department of Surgery, Baylor College of Medicine, Houston,TX 77030

Received May 30, 1990

To elucidate some of the early mechanisms underlying the response of primary human endothelial cells to the initiation of flow, we investigated the changes in inositol lipid metabolism in cells exposed to arterial and venous levels of shear stress. We used a radioimmunoassay specific for inositol-1,4,5-trisphosphate (Ins1,4,5P_3) to demonstrate that initiation of an arterial shear stress caused a rapid rise in Ins1,4,5P_3 levels which peaked after approximately 30 seconds of flow (2.1±0.2 fold stimulation) and remained elevated for at least 6 minutes after the initiation of flow. This increased Ins1,4,5P_3 concentration is similar in magnitude to the increase caused by 10 μM histamine (2.8±0.3 fold stimulation). Thus these cells may detect the presence of mechanical stress by a signal transduction pathway involving inositol lipid metabolism.

The force exerted by the flowing blood on the endothelial cells which line the vasculature causes these cells to respond in certain specific ways. Changes in morphology(1,2), arachidonic acid metabolism(3-6) and protein synthesis(7) have been described in studies using cultured primary human umbilical vein endothelial cells subjected to arterial levels of steady shear stress. The metabolic studies have demonstrated that endothelial cells respond quite selectively to the initiation of mechanical shear stress. There is increased incorporation of arachidonic acid only into the phosphatidylinositol fraction of the membrane phospholipids and there is a selective production of prostacyclin relative to other eicosanoid products(6). These cells also increase their synthesis and release of tissue plasminogen activator (tPA) but not of plasminogen activator inhibitor, type 1 (PAI-1) when stimulated with flow(7). However, the mechanism by which these cells detect the presence of fluid flow in order to generate these responses remains to be elucidated.

The specific nature of the response to flow appears to indicate that shear stress stimulates the production of intracellular second messengers rather than causing a general perturbation of cellular metabolism. One such second messenger which may

*To whom correspondence should be addressed.

0006-291X/90 $1.50

be involved in mediating the response of endothelial cells to shear stress is inositol-(1,4,5) trisphosphate (Ins1,4,5P_3). An elevated level of intracellular Ins1,4,5P_3 has been identified as one of the earliest events in the response of endothelial cells to agonist stimulation such as bradykinin (8-10), histamine (12-16), thrombin (11-14,17) and endothelial cell growth factor (18). Ins1,4,5P_3 is generated by the activation of a specific phospholipase C which breaks down the membrane phospholipid phosphatidylinositol-4,5 bisphosphate (PtIns4,5P_2) (19-22). The products of phospholipase C activity are diacylglycerol (DAG), which remains in the plasma membrane, and Ins1,4,5P_3, which is released into the cytosol. Elevated levels of Ins1,4,5P_3 can bind to specific sites on the endoplasmic reticulum which can cause release of calcium from intracellular stores (23,24). Elevated levels of intracellular calcium in conjunction with elevated levels of diacylglycerol in the plasma membrane may result in the activation of protein kinase C (25).

To investigate the signal transduction events which occur in endothelial cells stimulated with shear stress, we used a radioimmunoassay (RIA) specific for Ins1,4,5P_3 to determine the intracellular level of this second messenger. By comparing the level of Ins1,4,5P_3 in cells exposed to arterial and venous levels of shear stress with cells which are maintained in stationary culture we can determine if inositol lipid metabolism is enhanced. As an additional control, we compare the shear stress stimulated cells with cells which have been exposed to histamine, a substance known to increase inositol lipid metabolism.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were harvested from fresh umbilical cords using the methods adapted from Gimbrone (26). Briefly, the veins were cannulated, rinsed with approximately 100 ml phosphate buffer (consisting of 0.15M NaCl, 0.4 mM KCl, 10mM glucose, 0.2M NaH_2PO_4, and 0.1M Na_2HPO_4), and filled with collagenase solution (55 U/ml) in phosphate buffered saline (Gibco). The collagenase solution was allowed to incubate in the vein for 30 minutes at room temperature. The vein was then rinsed with 100 ml phosphate buffer and the effluent was collected and centrifuged for 10 minutes at 700g. The cell pellet was resuspended in complete medium containing M199 (Gibco) with 20% heat inactivated iron supplemented newborn calf serum (Hyclone) with 1% antibiotics (PSN) and 300mg/ml L-glutamine. The cells from 4 to 6 umbilical cords were pooled and seeded onto glass slides (Fisher) at an initial density of 10^4 cells/cm^2. The slides had been previously washed in a mild detergent, soaked in 0.5M sodium hydroxide for 2 hours, then thoroughly rinsed with deionized water. The slides with the attached endothelial cell monolayer were placed in 100mm non-tissue culture grade petri dishes with 10ml complete medium. The cells were kept at 37C in a humidified incubator with 5% CO_2. Slides were used for experiments after the cells had reached confluence, typically 3 to 5 days after seeding.

Flow chamber

Confluent monolayers of primary endothelial cells were exposed to mechanical stress in a machined polycarbonate flow chamber designed to give a parallel flat plate flow geometry (3,5). With this chamber the entire monolayer is exposed to the same well defined level of shear stress. The dimensions of the flow chamber were 2.5cm

wide (perpendicular to the direction of flow) and 6.4cm long (parallel to the direction of flow) resulting in an area of cell monolayer exposed to flow of 16 cm^2 which corresponds to approximately 10^6 cells. The gap width between the glass slide and the flow chamber was 220 microns. The glass slide with the attached endothelial cell monolayer was mounted on the flow chamber and was held in place by an applied vacuum. The flow rate of medium through the flow chamber was controlled by a constant head recirculating flow loop (3). The flow loop consisted of an upper and lower reservoir with the flow chamber situated between them. The flow rate (and consequently the shear stress) was determined by the hydrostatic pressure head due to the vertical distance between the upper and lower reservoirs. Medium was recirculated from the lower reservoir to the upper reservoir by a roller pump. The entire apparatus was maintained at 37C by two air curtain incubators and the medium was gassed with a mixture of 95% air and 5% CO_2 to maintain pH. The endothelial cell monolayers were stimulated with two levels of shear stress, 22 dynes/cm^2 which is representative of an arterial shear stress and 2 dynes/ cm^2 which is representative of a venous shear stress.

Determination of inositol (1,4,5) trisphosphate

The endothelial cell monolayer was washed three times with M199 (without serum) and the medium was changed to M199 (without serum) 12 - 24 hours prior to the experiment. Then 1 hour before the experiment the slides were photographed and subsequently counted to determine the cell density. Initially, the slide was perfused with M199 at 0.07 dynes/cm^2 for 30 minutes. This low level of shear stress was delivered by a syringe pump (Harvard Apparatus). After this equilibration time the shear stress was increased to either 2 or 22 dynes/cm^2 for times from 30 seconds to 15 minutes. After exposure to the elevated shear stress the slide was quickly removed from the flow chamber and the cells were lysed by adding 1ml ice cold 10% trichloroacetic acid (TCA) and thoroughly scraping the slide. The cell lysate was then immediately frozen in liquid nitrogen and was stored at -20C for subsequent analysis. The samples were thawed at room temperature and centrifuged for 10 minutes at 500g. The supernatant was transferred to a glass test tube with 10ml ethyl ether. This mixture was vigorously mixed for 15 seconds then allowed to stand for 2 minutes to allow the water and ether phases to separate. The ether phase was decanted and the extraction was repeated two more times. Finally, the pH of the water phase was adjusted to 7.4 with sodium bicarbonate. The efficiency of the extraction procedure was found to be 80% as determined by extracting radioactively labeled Ins1,4,5P_3 or by using an RIA to determine the total mass of Ins1,4,5P_3. The amount of Ins1,4,5P_3 in each sample was determined by an RIA system supplied by Amersham. The cross reactivity of the binding protein with other compounds, including other phosphorylated inositides, is small, less than 0.5%. However, for inositol 1,3,4,5 tetrakisphosphate the cross reactivity was 6.4%. The cross reactivity with the components of M199 or of the TCA solution was minimal.

Histamine stimulation

Confluent monolayers of endothelial cells seeded on glass slides were washed with M199 24 hours prior to the experiment. Before addition of the agonist the monolayers were photographed to determine the cell density. The medium in the petri dish with the slide was aspirated and replaced with medium containing 10 μM histamine (Sigma). The petri dish was then returned to the incubator for either 30 seconds or 4 minutes. After incubation with the agonist, the cells were lysed and the Ins1,4,5P_3 level was determined as described above.

Results

Primary human umbilical vein endothelial cells which were maintained in stationary culture conditions (0 dynes/cm^2) had a resting level of Ins1,4,5P_3 of 3.6± 0.4

Table 1

Resting level and histamine stimulation of Ins1,4,5P3 levels in primary HUVEC cultures

	stationary control	30 seconds 10 μM histamine	4 minutes 10 μM histamine	30 minutes** 0.07 dynes/cm^2
Ins1,4,5P3 concentration pmol/10^6 cells	3.6 ± 0.4(6)	10.2 ± 1.0(3)	4.6 ± 0.4(3)	3.2 ± 0.4(6)
fold stimulation	1.0	2.8 ± 0.3*	1.3 ± 0.2	1.0

The level of Ins1,4,5P$_3$ in primary human umbilical vein endothelial cells was determined by RIA for cells which had been maintained in an incubator under stationary culture conditions (0 dynes/cm^2). Cells were stimulated with 10μM histamine for the time periods indicated as described in the text. Results are presented as mean ± SEM. The number of separate cord pools used is indicated by the number in parenthesis. The * indicates a significant increase in Ins1,4,5P$_3$ relative to control ($p < 0.05$ by students t-test for paired data). ** The slides with the HUVEC monolayer were mounted on the flow chamber and perfused for 30 minutes at 0.07 dynes/cm^2. The level of Ins1,4,5P$_3$ in these cells was not significantly different from control slides which had been maintained in an incubator.

(mean ± SEM) pmol/10^6 cells. This value was obtained from 6 separate umbilical cord pools and normalized for the extraction efficiency as described above.

The response of these cells to histamine stimulation is summarized in table 1. Within 30 seconds exposure to 10 μM histamine the intracellular concentration of Ins1,4,5P$_3$ had increased to 2.8 ± 0.3 times the control levels. By 4 minutes exposure to 10 μM histamine the level of Ins1,4,5P$_3$ had decreased to 1.3 ± 0.2 times the control levels.

In order to control for any possible mechanical stimulation of the endothelial cell monolayer in the process of mounting the glass slide onto the flow chamber, the cells were perfused at a low level of shear stress (0.07 dynes/cm2) for the first 30 minutes. After this equilibration time the Ins1,4,5P$_3$ level was not significantly different from its basal value (table 1). The effect of increasing the shear stress to either 2 or 22 dynes/cm^2 on the concentration of Ins1,4,5P$_3$ is shown in figure 1. At arterial stress levels (22 dynes/cm^2) there was a rapid increase in intracellular Ins1,4,5P$_3$ which peaked at approximately 30 seconds exposure to flow at 2.1 ± 0.2 fold stimulation. After this initial peak the level of Ins1,4,5P$_3$ remained significantly elevated for at least 6 minutes (1.6 ± 0.3 fold stimulation) in cells exposed to 22 dynes/cm^2 shear stress. Monolayers exposed to a venous level of shear stress (2 dynes/cm^2) had a much smaller increase in intracellular Ins1,4,5P$_3$ concentration.

Discussion

We have utilized an RIA specific for Ins1,4,5P$_3$ to show that the initiation of hemodynamic shear stress can cause increases in the intracellular concentration of

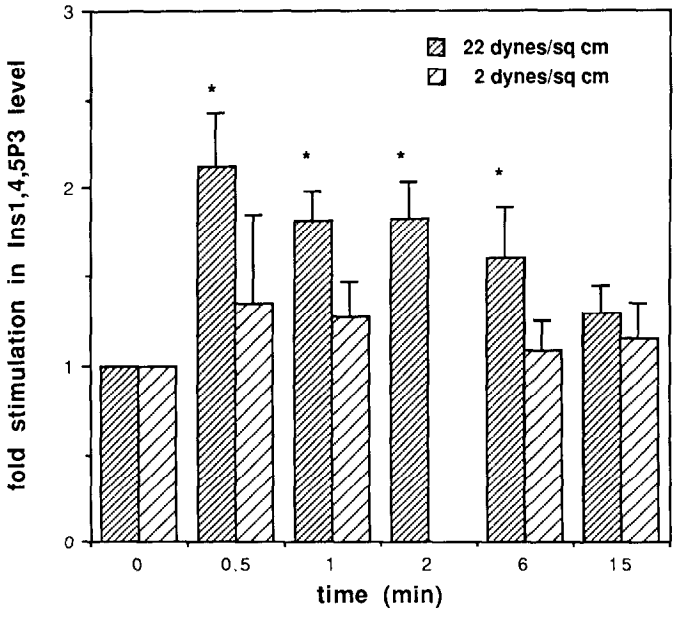

Figure 1: The intracellular level of $Ins1,4,5P_3$ in primary HUVEC exposed to shear stress of either 2 or 22 dynes/cm^2. Cells were exposed to 0.07 dynes/cm^2 for 30 minutes prior to the increase in shear stress. Each value represents the mean of results from 3 to 6 separate umbilical cord pools. The * indicates that cells exposed to 22 dynes/cm^2 for 0.5, 1, 2, and 6 minutes had a significantly greater $Ins1,4,5P_3$ levels ($p < 0.05$ by students t-test for paired samples). No $Ins1,4,5P_3$ was detected in the media after exposure to cells.

this second messenger in primary HUVEC. Furthermore, the shear stress induced increase in the $Ins1,4,5P_3$ level is similar in magnitude, though slightly smaller than the increase caused by the inflammatory mediator histamine.

Shear stress stimulated endothelial cells exhibit a rapid rise in $Ins1,4,5P_3$ level which peaks approximately 30 seconds (figure 1) after the initiation of an arterial level of shear stress (22 dynes/cm^2). In contrast to the histamine induced rise in $Ins1,4,5P_3$ concentration, however, the mechanical stress induced increase in $Ins1,4,5P_3$ remains elevated for several minutes after the increase in shear stress from 0.07 dynes/cm^2 to 22 dynes/cm^2. This continued elevation of the $Ins1,4,5P_3$ level in response to a stimulus appears to be unique to shear stress stimulation since with other agonists such as histamine, thrombin, and bradykinin the $Ins1,4,5P_3$ level returns to near basal levels within 2 to 4 minutes after exposure to the agonist. In fact the time course of the response for the shear stress stimulated rise in $Ins1,4,5P_3$ level is quite similar to the time course of shear stress stimulated prostacyclin (PGI_2) synthesis(3,5). After initiation of flow, PGI_2 synthesis exhibits a burst of production followed by a steady state production rate which is lower than the initial burst, but higher than the rate of PGI_2 production of cells maintained in stationary culture conditions.

We have demonstrated the increased rate of arachidonic acid uptake and metabolism in shear stress stimulated endothelial cells (6). Our findings showed that one of the initial responses to flow was an increased incorporation of arachidonic acid into phosphatidylinositol and into diacylglycerol. The increased incorporation into phosphatidylinositol persisted for up to two hours while the peak level of incorporation into diacylglycerol occurred earlier than 30 minutes exposure to flow. The mechanism of this increased turnover of membrane bound arachidonic acid could involve the activation of phospholipase C or phospholipase A_2. Both of these mechanisms are present in stimulated endothelial cells (27). The results presented here indicate that shear stress activates a phosphatidylinositol specific phospholipase C which generates Ins1,4,5P_3 and diacylglycerol.

Previous investigators have studied signal transduction events in HUVEC by pre-labeling cells with [^{3}H]myo-inositol and observing the redistribution of the radioactive label when the cells were stimulated with an agonist. This procedure can give detailed information about the agonist induced rapid changes in inositol lipid metabolism including the depletion of PtIns4,5P_2 and the increases in Ins1,4,5P_3. However, this method is not particularly sensitive and requires large numbers of cells (typically 4 X 10^6 cells) to generate detectable quantities of radioactivity in all intracellular stores of labeled inositol. These studies on primary and passaged HUVEC have demonstrated that histamine will increase Ins1,4,5P_3 levels by 1.5 to 2.5 times control levels. Brock and Capasso (12) found a 1.6 fold stimulation in cells stimulated with 10 μM histamine for 15 seconds. Pollock et al. (14) found a 2.4 fold stimulation 30 seconds after a 20 μM stimulation and Halldorsson et al. (13) found a 1.7 fold increase after a 20 second stimulation with 5.5 μM histamine. Our results, obtained with an RIA system, are in agreement with these published values. Thus we conclude that this RIA system is detecting the same changes in inositol lipid metabolism as would be detected with the [^{3}H]inositol procedure.

In conclusion, we have shown that one of the earliest responses of human endothelial cells to increases in shear stress is the activation of a phospholipase C pathway. This results in increased levels of the second messenger Ins1,4,5P_3 which may mediate the signal transduction events in the endothelial cell response to the presence of mechanical stress.

Acknowledgments

We thank Lydia Sturgis for assistance with the flow loop and tissue culture and Dr. Elizabeth Hall for helpful discussions and advice. This research was partially supported by the National Institutes of Health grants HL-17437, HL-18762 and NS-18494 and grant C-938 from the Robert Welch Foundation.

References

1) Ives, C.L., Eskin,S.G. and McIntire, L.V. (1986) In Vitro Cell. Dev. Biol. 22, 500
2) Levesque, M.J. and Nerem R.M. (1985) J. Biomech. Engr. 107, 341-347

3) Frangos, J.F., Eskin, S.G., McIntire, L.V. and Ives, C.L. (1985) Science 227, 1477 - 1479
4) Grabowski, E.F., Jaffe, E.A. and Weksler, B.B. (1985) J. Lab. Clin. Med. 105, 36-43
5) Frangos, J.F., Eskin, S.G. and McIntire, L.V. (1988) Biotech. Bioeng 32, 1053-1060
6) Nollert, M.U., Hall, E.R., Eskin, S.G. and McIntire, L.V. (1989) Biochim. Biophys. Acta 1005, 72-78
7) Diamond, S.L., Eskin, S.G., and McIntire, L.V. (1989) Science 243, 1483-1485
8) Lambert, T.L., Kent, R.S. and Whorton, R. (1986) J. Biol. Chem. 261(32), 15288-15293
9) Voyno-Yasenetskaya, T.A., Tkachuk, V.A., Cheknyova, E.G., Panchenko, M.P., Grigorian, G.Y., Vavrek, R.J., Stewart, J.M. and Ryan, U.S. (1989) FASEB J. 3, 44-51
10) Kaya, H., Patton, G.M. and Hong, S.L. (1989) J. Biol. Chem. 264(9), 4972-4977
11) Lampugnani, M.G., Pedenovi, M., Dejana, E., Rotilio, D., Donati, M.B., Bussolino, F., Garbarino, G., Ghigo, D. and Bosia, A. (1989) Thromb. Res. 54, 75-87
12) Brock, T.A. and Capasso, E.A. (1988) J. Cell. Physiol. 136, 54-62
13) Halldorsson, H., Kjeld, M. and Thorgeirsson, G. (1988) Arteriosclerosis 8(2), 147-154
14) Pollock, W.K., Wreggett, K.A. and Irvine, R.F. (1988) Biochem. J. 256, 371-376
15) Resink, T.J., Grigorian, G.Y., Moldabaeva, A.K., Danilov, S.M., and Buhler, F.R. (1987) Biochem. Biophys Res. Comun. 144(1), 438-446
16) Voyno-Yasenetskaya, T.A., Grigorian, G.Y., Danilov, S.M., Resink, T.J., Tkachuk, V.A., and Repin, V.S. (1989) J. Mol. Cell Cardiol. 21(Supp. 1), 145-149
17) Carter, A.J., Eisert, W.G. and Muller, T.H. (1989) Thromb. Haemo. 61(1), 122-126
18) Moscat, J., Moreno, F., Herrero, C., Lopez, C. and Garcia-Barreno, P. (1988) Proc. Natl. Acad. Sci. USA 85, 659-663
19) Rhee, S.G., Suh, P., Ryu, S. and Lee, S.Y. (1989) Science 244, 546-550
20) Berridge, M.J. and Irvine, R.F. (1989) Nature 341, 197-205
21) Majerou, P.W., Connolly, T.M., Deckmyn, H., Ross, T.S., Bross, T.E., Ishii, H., Bansal, V.S., Wilson, D.B. (1986) Science 234, 1519-1526
22) Nishizuka, Y. (1984) Science 225, 1365-1370
23) Ehrlich, B.E. and Watras, J. (1988) Nature 336, 583-586
24) Meyer, T., Holowka, D. and Stryer, L. (1988) Science 240, 653-655
25) Berridge, M.J. (1987) Ann. Rev. Biochem. 56, 159-193
26) Gimbrone, M.A. (1976) Prog Hemostas. Thromb. 3, 1-28
27) Hong, S.L. and Deykin, D. (1982) J. Biol. Chem 257, 7151-7154