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MECHANISM OF SHEAR-INDUCED PROSTACYCLIN PRODUCTION IN ENDOTHELIAL CELLS

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SUMMARY: Human umbilical vein endothelial cells at confluence were subjected to steady shear flow. It was previously shown that flow induced a burst in prostacyclin production followed by a sustained stimulation of production several fold higher than basal levels (1). In the presence of EGTA, prostacyclin production was inhibited in the steady state phase by 74%. Preincubation of endothelial cells with quin2/AM, used here as an intracellular calcium chelator, also inhibited the production of prostacyclin (83%). Inhibition of intracellular calcium mobilization had no significant effect. Incubation of cells with nifedipine, a voltage operated channel blocker, had no effect on shear induced prostacyclin production, whereas ibuprofen decreased shear induced prostacyclin production. RHC-80267, a diacylglycerol lipase inhibitor, inhibited 66% of shear induced PGI_2 production. Our results suggest that both extracellular and intracellular Ca^{2+} are necessary and the phospholipase C pathway may be the main source for liberating arachidonic acid in shear induced prostacyclin production.

Vascular endothelial cells form a multifunctional lining of the intimal surface of blood vessels. This lining is continuously subjected to both steady and pulsatile fluid stresses resulting from the flow of blood. Some of the physiological roles of hemodynamic shear are flow dependent dilation and flow dependent growth of vessel diameter (2). Hemodynamics have also been

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ABBREVIATIONS
Prostacyclin (PGI_2); human umbilical vein endothelial cells (HUVEC); arachidonic acid (AA); EGTA (ethylene glycol bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid); TMB-8 (8-N,N-(diethylamino) octyl-3,4,5-trimeth-oxybenzoate); quin2/AM (2-{[2-bis(carboxymethyl)-amino-5-methyl-phenoxy]-methyl}-6-methoxy-8-bis-carboxymethyl)-amino-quinoline); RHC-80267 (1,6-di (O-(carbamoyl) cyclohexanone oxime) hexane).

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implicated in some disease processes such as atherogenesis and hypertension (3,4). Furthermore, in vitro studies have shown that fluid shear can alter several cell functions such as prostacyclin (PGI_2) production (1,5), histamine forming capacity (6) and fluid phase endocytosis (7). Despite the numerous examples of the effect of hemodynamic shear on the endothelium, the mechanism of action of shear has yet to be elucidated.

The effect of shear stress on the production of prostacyclin in cultured human endothelial cells has previously been reported (1). Unlike other agonists of prostacyclin production, shear evokes a continuous stimulation of endothelial cells for several hours after the onset of flow. In addition, the steady state rate of prostacyclin production increases with increasing shear stress and pulsatility (1,5). In this communication, we provide evidence that both extracellular and intracellular calcium are essential for long term shear induced PGI_2 production in cultured HUVEC. We further demonstrate that preincubation of cells with RHC-80267, a diacylglycerol lipase inhibitor, decreases shear-induced prostacyclin production.

MATERIALS AND METHODS

Materials: 6-{5,8,9,11,12,14,15(n)-^{3}H} keto prostaglandin $F_{1\alpha}$ was purchased from New England Nuclear, Boston, MA. Tissue culture media and supplements were obtained from Grand Island, NY; collagenase enzyme from Worthington Biochemical, NJ; fetal bovine serum from Hyclone, Logan, UT; penicillin, and streptomycin from Gibco, Chagrin Falls, OH; trypsin-EDTA, gelatin, trypan-blue, ibuprofen, and EGTA from Sigma Chemical Company, St. Louis, Mo; nifedipine, TMB-8, quin2/AM were from Calbiochem, San Diego, CA. RHC-80267 was generously provided by Dr. Charles Sutherland of Rorer Central Research, Horsham, PA.

Cell Culture: Isolation and culture of human umbilical vein endothelial cellswas performed as described previously (1). Cell suspensions were seeded onto glass slides (75 mm x 38 mm) that had been treated earlier with 2% gelatin for 10 minutes and rinsed with PBS. Two slides were seeded per cord (5.0×10^4 to 1.0×10^5 cells/slide). Cultures became confluent after three or four days and experiments were run only with primary cells, 2 or 3 days after the cultures reached confluency.

Apparatus for Induction of Shear Stress: To simulate the hemodynamic environment of the circulation and to examine the effect of shear stress on the endothelium, we used the same flow apparatus as described earlier (1,5). The circuit was primed with 15 ml of culture medium, and a slide with a monolayer of cultured cells was positioned to form one of the plates in parallel plate chamber. Monolayers of endothelial cells were subjected to a mean shear-stress of 22 dynes/cm^2.

Experimental Design: The inhibitors used in this study act at different points of the arachidonic acid cascade. To study the effect of extracellular calcium, medium containing 2 mM EGTA was

used. To determine the role of intracellular calcium, cells were preincubated in 30 μM quin2/AM for 30 minutes and the experiment was carried out in normal quin2 free medium. To study the effect of nifedipine (20 μM), ibuprofen (10 μM), RHC-80267 (10 μM) and TMB-8 (10 or 100 μM), cells were preincubated for 30 minutes with the inhibitor and the experiments were carried out with their respective inhibitor-containing medium. Samples (1 ml each), were collected at different intervals and the flow circuit was simultaneously replenished with the same volume of fresh medium to maintain a constant circulating volume of 15 ml. At the end of each experiment (6 to 7 hours) the cells were detached by trypsinization and counted by hemocytometer to determine the total viable count, as determined by trypan-blue exclusion staining. Comparison of the light micrographs of the cell monolayers before and after each experiment revealed no significant changes in cell morphology. Prostacyclin was assayed by radioimmunoassay of the stable hydrolysis product 6-keto-$PGF_{1\alpha}$. Cumulative production was determined by performing a prostacyclin mass balance over the flow loop, taking into account samples withdrawn and medium replenished. The steady state production rate of PGI_2 was determined by fitting the cumulative production to a four-parameter equation by nonlinear regression analysis (1,5).

RESULTS AND DISCUSSION

To determine the role of intracellular free calcium, cells were preincubated with 30 μM quin2/AM for 30 minutes. Quin2/AM, being membrane permeable, is rapidly transported across biological membranes, where it is hydrolyzed by intracellular esterases. The resulting carboxylate form of quin2, being impermeable, remains trapped intracellularly, where it binds to intracellular calcium. At sufficiently high concentrations, quin2 has been shown to antagonize increases in intracellular free calcium (8). The production of PGI_2 by the cells exposed to quin2 was significantly inhibited (Table 1, Fig. 1). This indicates that elevated levels of intracellular calcium are necessary for shear induced prostacyclin production. The increase in intracellular free calcium may be due to the mobilization of extracellular Ca^{2+} into cytosol and/or the mobilization from intracellular Ca^{2+} storage pools. In the absence of extracellular calcium (2 mM EGTA), long term prostacyclin production was inhibited 74%. Interestingly, we found TMB-8, an inhibitor of intracellular calcium mobilization, had no significant inhibitory effect on long-term production (Table 1) suggesting that there may be little mobilization of calcium from intracellular stores. The minor inhibitory effect of TMB-8 at 100 μM we observed may be due to the inhibitors's nonspecificity (9,10). These results indicate that extracellular calcium is largely responsible for the elevated intracellular calcium levels.

Table 1. The effect of inhibitors on the long term shear-induced prostacyclin production rate

	pg $PGI_2/10^6$/ cells/min	% Change
Control	43± 2 (3)	
EGTA (2 mM)	11± 6 (6)	-74*
Control	129±11 (3)	
quin2/AM (30 μM)	22± 6 (4)	-83*
Control	282±23 (3)	
Ibuprofen (10 μM)	57±32 (3)	-80*
Control	121±11 (2)	
Nifedipine (20 μM)	125±27 (4)	+3NS
Control	88±14 (2)	
TMB-8 (10 μM)	89±23 (3)	+2NS
Control	90±28 (2)	
TMB-8 (100 μM)	58±31 (2)	-36NS
Control	120± 8 (3)	
RHC-80267 (10 μM)	41±10 (6)	-66*

*$p < 0.001$
NS = Not significant
Values are mean ± SD (n)

To determine the mode of entry of calcium, we studied the effect of nifedipine, a voltage operating Ca^{2+} channel blocker, on shear-induced PGI_2 production. The PGI_2 production was not altered (Table 1) indicating that the voltage operating Ca^{2+} channels were not involved in the mobilization of extracellular Ca^{2+} during shear stress. Recently, Colden-Stanfield et al (11) reported the absence of voltage operating calcium channels in endothelial cells. It therefore appears that calcium enters the cell by another, as-yet undetermined channel.

In endothelial cells, the major sources of AA are the hydrolysis of phospholipids by phospholipase A_2 and the sequential action of phospholipase C and diacylglycerol lipase. RHC-80267, a diacylglycerol lipase inhibitor, was used to determine whether the latter pathway was the source of AA for prostacyclin synthesis. Sutherland and Amin (12) reported that RHC-80267 specifically inhibited platelet diacylglycerol lipase (I_{50} - 4 μM), showing only a minor inhibitory effect on phospholipases A_2 and C. Others have reported RHC-80267 had no effect at a concentration of 10 μM in

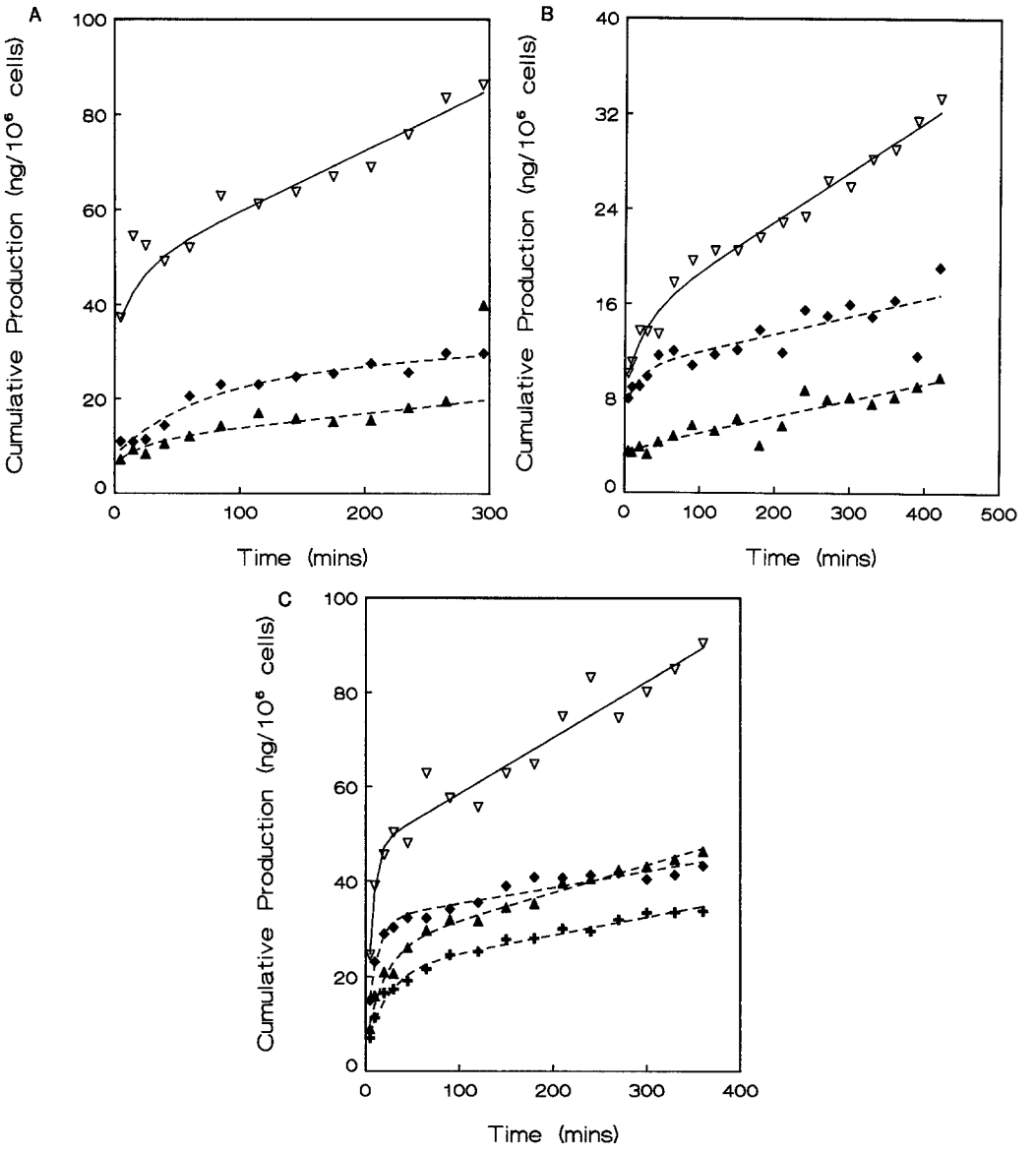

Fig. 1. Effect of quin2/AM, EGTA and RHC-80267 on the cumulative production of prostacyclin during 22 dynes/cm^2 of steady shear stress. (A) The replicate cell monolayers were incubated with 30 μm quin2/AM containing medium for 30 mins, then washed and exposed to shear (---). (B) Cells were exposed to shear in the presence of 2 mM EGTA (---). (C) Cells were incubated with medium containing 10 μm RHC-80267 for 30 mins and then exposed to shear with medium containing RHC-80267 (---). The controls (___) were exposed to shear in the presence of normal medium. The cumulative production data are shown with the fitted curve. The steady state production rate is taken as the slope of the curve at long times.

intact platelets, while at high concentrations (250 μM) this inhibitor was not specific, inhibiting other lipases (13,14). In the present study, however, 10 μM RHC-80267 was found to inhibit 66% of shear induced PGI_2 production. This suggests that

diacylglycerol lipase is involved in the release of AA during shear and that diacylglycerol (through the action of a phospholipase C) is being formed. The activation of phosphatidylinositol-specific phospholipase C in endothelial cells by humoral agonists such as bradykinin, histamine and thrombin has been observed (15,16). Dudley and Spector (17) have reported that phosphatidylinositol turnover provides AA for PGI_2 synthesis in thrombin, histamine and bradykinin stimulated human endothelial cells and RHC-80267 reduces the agonist stimulated release of PGI_2 by as much as 80%. The latter finding is quite consistent with the results presented here.

Incubation of endothelial cells with the cyclooxygenase inhibitor ibuprofen resulted in a 80% decrease in shear induced PGI_2 production. This suggests the point of action of shear stress is at the cyclooxygenation of AA or at preceding steps. Since it is generally believed that AA metabolism is rate limited by its availability (18), it is probable that shear stress acts by increasing the rate of release of AA.

From this study we conclude that both extracellular and intracellular calcium levels play an important role in shear-induced PGI_2 production. Extracellular Ca^{2+} is not mobilized through voltage operated calcium channels but enters by other means. The mechanism of action of shear stress is at the level of AA release or cyclooxygenase activation. In addition, our data indirectly indicate that the phospholipase C pathway may be important for the release of AA during shear stress. These results suggest that shear-induced stimulation of human endothelial cells may occur via a signal transduction mechanism similar to that of humoral agonists. Our future work will focus on the role of different phospholipids and inositol phosphates on shear-induced stimulation of endothelial cells.

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REFERENCES

1. Frangos, J.A., Eskin, S.G., McIntire, L.V., and Ives, C.L., Science, 227:1477-1479 (1985).
2. Langille, B.L., O'Donnell, F., Science, 231:405-407 (1986).
3. Fry, D.L., Circulation Res., 22:165-197 (1968).
4. Caro, G.G., Parker, K.H., Fish, P.J., and Lever, M.J., Clin. Hemorheol., 5:849-871 (1985).
5. Frangos, J.A., McIntire, L.V., and Eskin, S.G., Biotech. Bioeng., (in press).

6. DeForrest, J.M. and Hollis, T.M., Am. J. Physiol., 234:H701-H705 (1978).
7. Davies, P.F., Dewey, C.F., Jr., Bussolari, S.R., Gordon, E.J., and Gimbrone, M.A., Jr., J. Clin. Invest., 73:1121-1129 (1984).
8. Simon, M.F., Chap, H., and Douste-Blazy, L., Biochim. Biophys. Acta, 875:157-164 (1986).
9. Brotherton, A.F.A, and Hoak, J.C. Proc. Natl. Acad. Sci. U.S.A., 79:495-499 (1982).
10. Simpson, A.W.M., Hallam, T.J., and Rink, T.J. FEBS Lett. 176:139-143 (1984).
11. Colden-Stanfield, M., Schilling, W.P., Ritchie, A.K., Eskin, S.G., Navarro, L.T., and Kunze, D.L., Circulation Res., 61:632-640 (1987).
12. Sutherland, C.A., and Amin, D., J. Biol. Chem., 257:14006-14010 (1982).
13. Bross, T.E., Prescott, S.M., and Majerus, P.W., Biophys. Res. Commun., 116:68-74 (1983).
14. Oglesby, T.D., and Gorman, R.R., Biochem. Biophys. Acta, 793:269-277 (1984).
15. Hong, S.L., and Deykin, D. J. Biol. Chem., 257:7151-7154 (1982).
16. Halldorsson, H., Kjeld, M., and Thorgeirsson, G. Arteriosclerosis, 8:147-154 (1988).
17. Dudley, D.T., and Spector, A.A. Circulation, 74 (Suppl. II):231 (1986).
18. Irvine, R.F., Biochem. J., 204:3-16 (1982).