Endothelium-Derived Relaxing Factor Inhibits Thrombin-Induced Platelet Aggregation by Inhibiting Platelet Phospholipase C

By William Durante, Michael H. Kroll, Paul M. Vanhoutte, and Andrew I. Schafer

Endothelium-derived relaxing factor (EDRF) inhibits platelet function, but the mechanism underlying this inhibitory effect is not known. To examine this, cultured acetylsalicylic acid (ASA)-treated endothelial cells (EC) from bovine aorta (BAEC) or from human umbilical vein (HUVEC) were incubated with washed, ASA-treated human platelets. Incubation of platelets with either BAEC or HUVEC resulted in inhibition of thrombin-induced platelet aggregation that was dependent on the number of EC added. This effect was potentiated by superoxide dismutase and reversed by treating EC with N\textsuperscript{6}-nitro-L-arginine or by treating platelets with methylene blue, indicating that the inhibition of platelet aggregation was due to the release of EDRF by EC. EC significantly blocked the thrombin stimulated breakdown of phosphatidylinositol-4,5-bisphosphate (PIP\textsubscript{2}) and the production of phosphatidic acid in \textsuperscript{32}Porthophosphate-labeled platelets and of inositol trisphosphate in \textsuperscript{3}Hinositol-labeled platelets. In addition, the thrombin-mediated activation of protein kinase C (PKC) and phosphorylation of myosin light chain were inhibited in the presence of EC. Finally, thrombin stimulated an increase in cytosolic ionized calcium concentration ([Ca\textsuperscript{2+}]) in fura-2-labeled platelets that was abolished by concentration of EC which also blocked thrombin-induced aggregation. These data indicate that EDRF blocks thrombin-induced platelet aggregation by inhibiting the activation of PIP\textsubscript{2}-specific phospholipase C and thereby suppressing the consequent activation of PKC and the mobilization of [Ca\textsuperscript{2+}].

© 1992 by The American Society of Hematology.

EXPERIMENTAL

Materials. Human α-thrombin was obtained from US Biochemicals (Cleveland, OH); bradykinin, creatine phosphate (CP), creatine phosphokinase (CPK), Sepharose 2B-300, fatty acid-free bovine serum albumin (BSA), acetylsalicylic acid (ASA), superoxide dismutase (SOD), and methylene blue (MB) were from Sigma (St Louis, MO); EDTA and trypsin were from Gibco Laboratories (Grand Island, NY); [\textsuperscript{3}H]6-keto-PGF\textsubscript{1α} (153 Ci/mmol) were from New England Nuclear (Boston, MA); Dowex AG1-X8 anion exchange resin (formate form, 100-200 mesh) was from Bio-Rad Laboratories (Richmond, CA); D-Myo 1,2-[\textsuperscript{3}H]inositol (35 Ci/mmol/L) was from American Radiolabeled Chemicals (St Louis, MO); [\textsuperscript{3}H]acetoxyethyl ester was from Molecular Probes (Eugene, OR); high performance liquid chromatography (HPLC) grade organic solvents were from American Bioanalytical Chemicals (St Louis, MO); fura-2 acetoxymethyl ester was from Molecular Probes; Ethylene, OR; high performance liquid chromatography (HPLC) grade organic solvents were from American Radiolabeled Chemicals (St Louis, MO); fura-2 acetoxymethyl ester was from Molecular Probes (Eugene, OR); high performance liquid chromatography (HPLC) grade organic solvents were from American Radiolabeled Chemicals (St Louis, MO). Velamol silicone gel K5 plates were from Fisher Scientific (Fairlawn, NJ); N\textsuperscript{6}-nitro-L-arginine (L-NNA) was from Aldrich (Milwaukee, WI); and 6-keto-PGF\textsubscript{1α} antiserum was from Advanced Magnetics (Cambridge, MA).

EC culture. EC were obtained from human umbilical vein (HUVEC) or from bovine aorta (BAEC) and were grown as monolayers as previously described. Confluent monolayers of EC were preincubated with ASA (1 mmol/L) for 60 minutes, treated with trypsin (0.02%):EDTA (0.05%), and then collected by centrifugation at 450g for 10 minutes and suspended in Tyrode buffer.
Platelet preparation and aggregation. Whole blood from drug-free donors in 15% (vol/vol) acid-citrate-dextrose (ACD) was centrifuged at 220g for 14 minutes and the platelet-rich plasma (PRP) was collected and incubated with ASA (1 mmol/L) for 60 minutes. PRP was then acidified to pH 6.5 with AC, CP (5 mmol/L) and CPK (25 U/mL) added, and the PRP layered over a BSA density gradient and centrifuged at 1,600g for 15 minutes.2 Interface platelets were collected and subjected to repeat BSA density-gradient separation. Platelets were then gel-filtered through Sepharose 2B-300, collected in Tyrode buffer, and the platelet concentration adjusted to 3 x 10^10 platelets/mL (Coulter Counter Model ZF; Hialeah, FL).

Suspensions of washed platelets (450 µL) were incubated for 2 minutes in a Chronolog 480 VS aggregometer (Havertown, PA) with continuous stirring at 1,000 rpm and then stimulated with a submaximal concentration of thrombin (0.1 U/mL). Where indicated, EC in a volume of 5 to 50 µL were added to the platelet suspension 1 minute before thrombin stimulation. In some experiments SOD (60 U/mL) or bradykinin (10 nmol/L) was added to the platelet suspension immediately before the addition of EC. In other experiments EC were preincubated with L-NNa (100 µmol/L) for 60 minutes or, alternatively, washed platelets were pretreated for 30 minutes with methylene blue (10 µmol/L).

Platelet phospholipid turnover and protein phosphorylation. Washed platelets were radiolabeled with [3H]arachidonic acid as previously reported and suspended in Tyrode buffer at a concentration of 3 x 10^10 platelets/mL. For platelet lipid measurements the reactions were terminated at the designated time points by adding 3.75 x vol of ice-cold methanol/chloroform (2:1, vol/vol). Lipids were extracted according to the method of Bligh and Dyer and then separated by thin-layer chromatography using the procedure of Van Dongen et al. Lipid bands were detected by autoradiography, scraped, and counted for radioactivity. For platelet protein phosphorylation analysis, platelets were stimulated as described and the reactions stopped by the addition of a solution of 50% glycerol, 10% diethiothreitol, 8% sodium dodecyl sulfate (SDS), and trace bromphenol blue, followed by immediate boiling for 5 minutes. SDS-polyacrylamide gel electrophoresis was performed on 6% to 16% gradient slab gels as described by Laemmli and the radiophosphorylated proteins located by autoradiography.

Inositol trisphosphate (IP_3) measurement. Washed platelets were suspended in 2 mL of Tyrode buffer and incubated with [3H]myoinositol (200 µCi) at 37°C in a shaking water bath for 3 hours. Labeled platelets were then gel-filtered through Sepharose 3B-300, resuspended in Tyrode buffer containing LiCl (10 mmol/L) at a concentration of 3 x 10^10 platelets/mL, and then incubated for an additional 30 minutes before being used. For IP_3 measurements the reactions were stopped at various time points by adding 3.75 x vol of ice-cold methanol/chloroform (2:1, vol/vol). Chloroform (0.62 mL) and water (0.62 mL) were then added and the samples vortexed and centrifuged at 800g for 10 minutes. The upper phase containing the water-soluble inositol phosphates was removed and applied to a 1-mL Dowex AG1-X8 column. Inositol phosphates (IP_1, IP_2, IP_3) were separated as previously described by Berridge et al. Sodium tetraborate (5 mmol/L; 32 mL) was added to elute the free inositol and glycerophosphoinositol while IP_3 was eluted from the column with 50 mL of solution containing 0.2 mol/L ammonium formate and 0.1 mol/L formic acid. IP_1 and IP_2 were eluted with 30 mL of 0.4 mol/L ammonium formate–0.1 mol/L formic acid and 1 mol/L ammonium formate–0.1 mol/L formic acid solution, respectively. For IP_3 measurement, fractions (4 mL) were collected into vials and 12 mL of scintillation fluid added. The samples were then mixed and counted for radioactivity. Control studies using labeled IP_3 had shown that this elution pattern yields 90% recovery.

Cytoplastic ionized Ca^2+ ([Ca^2+]_i) measurement. Washed platelets were suspended in 1 mL of loading buffer (145 mmol/L NaCl, 10 mmol/L HEPES, 10 mmol/L MgCl_2, 6 mmol/L glucose, 5 mmol/L KCl; pH 7.35) and incubated with the acetoxymethyl ester of fura2 (2 µmol/L) at 37°C in a gently shaking water bath for 60 minutes. Fura2 loaded platelets were then gel-filtered through Sepharose 2B-300 and resuspended in loading buffer containing Ca^2+ (1 mmol/L) at a concentration of 1 x 10^9 platelets/mL. [Ca^2+] was measured in a Deltascan-1 double-beam fluorescence spectrophotometer (Photon Technology International, Bristol, CT) using 1.5-mL aliquots of the platelet suspension. [Ca^2+] was determined by the calibration procedure of Tsien et al using a kd for fura2 of 224 nm.

PGI_2, and cGMP radioimmunoassays. Production of PGI_2 was quantitated by radioimmunoassay of its stable breakdown product 6-keto-PGF_1α using a polyclonal rabbit antiserum as previously described. Platelet cGMP was determined in acetylated samples using a commercially available radioimmunoassay kit (New England Nuclear, Boston, MA).

RESULTS

Thrombin (0.1 U/mL) produces submaximal and irreversible aggregation of washed, ASA-pretreated platelets within 5 minutes. Incubation of platelets with increasing concentrations of ASA-pretreated BAEC (5 x 10^4 to 1 x 10^5 cells) for 1 minute causes increasing inhibition of thrombin-induced platelet aggregation (Fig 1A). The antiaggregatory effect of EC is also found when platelets are stimulated with collagen (data not shown). The possibility that PGI_2 is involved in the anti-aggregatory effect of EC is excluded because both cell types are pretreated with ASA and 6-keto-PGF_1α is undetectable (<40 pg/mL) in the platelet-EC mixtures, whereas platelet-EC mixtures in the absence of ASA pretreatment produce greater than 6,000 pg/mL 6-keto-PGF_1α after thrombin stimulation. The inhibition of platelet aggregation by EC is associated with an increase of platelet cGMP levels. Incubation of platelets with EC (1 x 10^6 cells) for 1 minute increases the cGMP content of platelets from 0.109 ± 0.008 to 0.174 ± 0.006 pmol/10^9 platelets (P < .05; Student’s unpaired t-test).

The EC-derived platelet inhibitory activity is characterized as EDRF on the basis of its previously documented pharmacologic profile. SOD (60 U/mL), which prolongs the half-life of EDRF, significantly potentiates the platelet inhibitory effect of EC (1 x 10^6 cells) (Fig 1B) while having no effect on thrombin-induced platelet aggregation in the absence of EC (data not shown). In contrast, incubation of EC with a specific inhibitor of EDRF synthesis, L-NNa (100 µmol/L), abolishes the antiaggregatory effect of EC (Fig 1B). In addition, treating platelets with methylene blue (10 µmol/L), which inhibits the vasorelaxant effect of EDRF, reverses the inhibition of platelet aggregation by EC (Fig 1B). Finally, if EC are stimulated with bradykinin (10 nmol/L) before thrombin addition the
A EC

Y mln

0 EC

Y 1 mln

C.
c

EC

DURANTE ET AL

Fig 1. Effect of EC on thrombin-stimulated platelet aggregation. Increasing numbers of BAEC (5 × 10^5 to 1 × 10^6 cells) produce increasing inhibition of platelet aggregation (A). The platelet inhibitory effect of BAEC (1 × 10^6 cells) was potentiated by superoxide dismutase (SOD; 60 U/mL), and was prevented by treating platelets with MB (10 μmol/L) or by treating BAEC with L-NNA (100 μm) (B). The platelet inhibitory effect of BAEC (1 × 10^5 cells) was potentiated by bradykinin (BK, 10 nmol/L) (C). Thrombin (0.1 U/mL) was added to stirred, washed platelet suspensions at the point of the arrow and the decrease in optical density followed for 5 minutes. Representative tracings are from one of five separate experiments.

platelet inhibitory effect of EC (1 × 10^5 cells) is significantly potentiated (Fig 1C); however, this is only observed with BAEC and not with HUVEC (data not shown).

Both BAEC and HUVEC inhibit platelet aggregation through their release of EDRF. The release of platelet inhibitory EDRF by HUVEC is dependent on the number of passages, with primary cells exerting the most potent platelet inhibitory EDRF activity (Fig 2). In addition, HUVEC that were not treated with ASA show a greater ability to inhibit platelet aggregation. In contrast to HUVEC, the release of platelet inhibitory EDRF by BAEC is unchanged for up to eight passages (data not shown). In subsequent biochemical experiments with HUVEC only primary cultures were used.

In [32P]orthophosphate-labeled platelets, thrombin (0.1 U/mL) induces a rapid breakdown and resynthesis of PIP_2, followed by the generation of phosphatidic acid (PA). Incubation of platelets with BAEC (1 × 10^6 cells) for 1 minute significantly attenuates the breakdown in PIP_2 and the subsequent formation of PA (Fig 3). The inhibition of PA production by BAEC depends on the number of EC present and correlates with the inhibition of platelet aggregation in the same platelet preparations (Fig 4). Furthermore, the inhibition of PA production by EC is potentiated by SOD and reversed by pretreating EC with L-NNA (data

Fig 2. Effect of passage number on platelet inhibitory EDRF release by ASA-treated (---) and non-ASA-treated (—) HUVEC. Data points represent the mean of one to three separate experiments.

Fig 3. Effect of EC on thrombin-stimulated breakdown of platelet PIP_2 (A) and phosphatidic acid (PA) production (B). Washed platelets were stimulated by thrombin (0.1 U/mL) in the presence (○) or absence (●) of BAEC (1 × 10^5 cells). PIP_2 and PA were measured synchronously in [32P]orthophosphate-labeled platelets as described in Experimental Procedures. Data points represent the mean of three separate experiments. Statistically significant (P < .05; Student’s unpaired t-test) effects by EC coincubation are denoted by an asterisk.
EDRF inhibits platelet activation

The decrease in platelet IP₃ formation by EC is associated with inhibition of platelet aggregation in the same platelet preparation (Fig 5; inset). Figure 6 shows that thrombin-induced phosphorylation of platelet p47 and p20 are significantly reduced in the presence of BAEC (1 x 10⁶ cells). Similar results are found with HUVEC (data not shown). Finally, the thrombin-stimulated increase of [Ca²⁺] in fura2-loaded platelets is abolished by incubating the platelets with concentrations of EC (1 x 10⁶ cells), which also prevents platelet aggregation (Fig 7). Comparable inhibition of thrombin-induced increases of platelet [Ca²⁺], is noted with BAEC and HUVEC.

**DISCUSSION**

In the present study we have shown that EDRF-mediated inhibition of platelet activation is associated with the inhibition of PIP₂-specific phospholipase C. This conclusion is based on data showing that EDRF blocks thrombin-induced breakdown of PIP₂ and the consequent production of IP₃ and PA. The inhibition of platelet phospholipase C activation by EDRF blocks subsequent intracellular signaling events: PKC activation (as measured by p47 phosphorylation) and increases of platelet [Ca²⁺],, (as measured by p20 phosphorylation and by direct measurements of [Ca²⁺], in fura2-loaded platelets) are inhibited by EDRF. The finding that EDRF prevents the thrombin-stimulated increase in platelet [Ca²⁺], is consistent with observations reported by Busse et al.²⁴

A similar inhibitory effect by EDRF on phospholipase C activation has been reported in vascular smooth muscle cells. Rapoport²⁵ and others²⁶,²⁷ have shown that EDRF-mediated inhibition of vascular smooth muscle contraction is associated with a decrease in phosphatidylinositol hydrolysis. Furthermore, as we have observed in platelets, EDRF can inhibit intracellular signaling events that follow phospholipase C activation. EDRF has been found to decrease

---

**Fig 4.** Correlation between EC concentration-dependent inhibition of platelet aggregation (A) and inhibition of phosphatidic acid (PA) production (B). Washed, [³²P]orthophosphate-labeled platelets were stimulated by thrombin (0.1 U/mL) (at the point of the arrow) in the presence of various concentrations of BAEC (9 to 1 x 10⁶ cells) and the decrease in optical density followed for 2 minutes at which time PA was measured as described in Experimental Procedures. Similar results were obtained in three separate experiments.

---

**Fig 5.** Effect of EC on thrombin-stimulated platelet inositol triphosphate (IP₃) production and platelet aggregation. Washed, [³H]myoinositol-labeled platelets were stimulated by thrombin (0.1 U/mL) in the presence (○) or absence (●) of HUVEC (1 x 10⁶ cells). IP₃ was measured as described in Experimental Procedures. Inset: Aliquots of washed platelets from the same [³H]myoinositol-labeled preparation were incubated in the presence (+EC) or absence (−EC) of HUVEC (1 x 10⁶ cells). Platelets were stimulated by thrombin (0.1 U/mL) at the point of the arrow and the decrease in optical density recorded.
[Ca^{2+}]	extsuperscript{30} and to reduce the incorporation of \textsuperscript{32}P into myosin light chain\textsuperscript{39} in smooth muscle cells. Thus, it appears that EDRF can inhibit both platelet aggregation and smooth muscle tone by its inhibitory effect on the same biochemical pathway of extracellular signal transduction.

The mediator of EDRF inhibition of phospholipase C may be cGMP. EDRF has been shown to activate soluble guanylate cyclase and increase cGMP levels in both platelets\textsuperscript{40,41} and vascular smooth muscle cells.\textsuperscript{41} In addition, lipophilic analogues of cGMP inhibit polyphosphoinositide hydrolysis and Ca\textsuperscript{2+} mobilization in thrombin-stimulated platelets\textsuperscript{42} and in smooth muscle cells.\textsuperscript{43,44} A recent study has shown that nitrovasodilators, which increase platelet cGMP levels,\textsuperscript{44} can also inhibit thrombin-induced platelet PA production.\textsuperscript{45} A role for cGMP in mediating the platelet inhibitory effect of EDRF in our study is substantiated by the observation that the anti-aggregatory effect of EDRF is associated with an increase in platelet cGMP levels and that the inhibitory effect of EDRF is suppressed by methylene blue, a potent inhibitor of soluble guanylate cyclase.\textsuperscript{39}

Although the exact mechanism by which cGMP regulates phospholipase C is not presently known, a recent report using vascular smooth muscle cells suggests that cGMP may inhibit the interaction between the guanine nucleotide binding regulatory protein and phospholipase C\textsuperscript{46}; however, this has not been confirmed in platelets.\textsuperscript{47}

Our study has also shown that EDRF released by EC from different species and vascular sources (HUVEC and BAEC) exert comparable inhibitory actions on platelet activation. Moreover, we found that passage of primary cultures of HUVEC results in a progressive loss of the capacity of these cells to produce EDRF and to inhibit platelet aggregation. This was not observed with BAEC. A similar passage-dependent decrease in HUVEC PGI\textsubscript{2} production has been previously reported\textsuperscript{48,49} and would suggest caution in using subcultured HUVEC when studying platelet inhibitory autacoid release. Finally, we have also shown that treating primary HUVEC cultures with ASA significantly reduces the ability of HUVEC to inhibit platelet aggregation. This reduction in anti-aggregatory effect by ASA treatment is associated with a complete blockade in PGI\textsubscript{2} production. Therefore, it appears that both EDRF and PGI\textsubscript{2} play an important role in modulating platelet aggregation. The finding that EDRF and PGI\textsubscript{2} act synergistically to inhibit platelet aggregation\textsuperscript{49} suggests that the dual release of these two autacoids from EC may play an important physiologic role in regulating platelet function.

In the present study thrombin and the shear stress of stirring may have increased the basal release of EDRF from EC. Thrombin has been found to stimulate the release of
EDRF\(^2\) and may have contributed to the anti-aggregatory action of the EC under our conditions. It is also known that the release of EDRF from EC is stimulated by the shear stress that develops in the stirring platelet suspension within the aggregometer cuvette.\(^4\) Bradykinin, a stimulus for EDRF release,\(^{51}\) significantly potentiates the anti-aggregatory action of BAEC, whereas the inhibitory effect of HUVEC was unaffected. This divergent response to bradykinin by the EC may reflect a species difference or a variation in cell culture procedure. HUVEC were grown as monolayers on plates and were collected by trypsin treatment just before their use while BAEC were grown on microcarrier beads and used several days after trypsin treatment. Because trypsin decreases the number of bradykinin receptors on EC,\(^{52}\) the lack of release of EDRF from bradykinin-stimulated HUVEC may be due to the loss of bradykinin receptors after trypsin treatment while the duration of culturing for BAEC after exposure to trypsin may have permitted the recovery of bradykinin receptors.

In summary, we have shown that the platelet anti-aggregatory effect of EDRF is due to the inhibition of platelet PIP\(_2\)-specific phospholipase C. This result supports the hypothesis that EDRF promotes blood fluidity by inhibiting platelet aggregation and vascular smooth muscle contraction through a functionally significant inhibitory effect on a common biochemical pathway of cellular activation.

REFERENCES


28. Schafer AI, Rodriguez R, Loscalzo J, Gimbrone MA Jr:
Inhibition of vascular endothelial cell prostacyclin synthesis by plasmin. Blood 74:1015, 1989
35. Rapoport RM: Cyclic guanosine monophosphate inhibition of contraction may be mediated through inhibition of phosphatidylinositol hydrolysis in the rat aorta. Circ Res 58:407, 1986
Endothelium-derived relaxing factor inhibits thrombin-induced platelet aggregation by inhibiting platelet phospholipase C

W Durante, MH Kroll, PM Vanhoutte and AI Schafer