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{o}-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}Hinosoinsitol-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 furall-loaded platelets that was abolished by concentrations 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+}].

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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]outradiophosphoric acid (5 mCi/mL) and \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/mmole/L) was from American Radiolabeled Chemicals (St Louis, MO); fura2 acetoxymethyl ester was from Molecular Probes (Eugene, OR); high performance liquid chromatography (HPLC) grade organic solvents were from American Chemicals (Muskegan, MI); Whatman silica gel K5 plates were from Fisher Scientific (Fair Lawn, NJ); N\textsuperscript{o}-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.
(130.0 mmol/L NaCl, 10.0 mmol/L Na citrate, 10.0 mmol/L Tris base, 9.0 mmol/L NaHCO₃, 6.0 mmol/L glucose, 3.0 mmol/L KCl, 1.0 mmol/L CaCl₂, 0.9 mmol/L MgCl₂, 0.8 mmol/L KH₂PO₄; pH 7.35) containing ASA (1 mmol/L). In experiments with BAEC, cells were seeded onto Cytodex-3 microcarrier beads (Pharmacia, Uppsala, Sweden), stirred until they were confluent (3 to 4 days), and used 60 minutes after the addition of ASA (1 mmol/L).

**Platelet preparation and aggregation.** Whole blood from drug-free donors in 15% (vol/vol) acid-citrate-dextrose (ACD) was centrifuged at 220 g 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 ACID, CP (5 mmol/L) and CPK (25 U/mL) added, and the PRP layered over a BSA density gradient and centrifuged at 1,600 g for 15 minutes. 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 10⁹ 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 [³²P]labeled phosphate as previously reported and suspended in Tyrode buffer at a concentration of 3 x 10⁹ platelets/mL. For platelet lipid measurements the reactions were terminated at the designated time points by adding 3.75 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% dithiothreitol, 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 ([I]P₃) 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 10⁹ platelets/mL, and then incubated for an additional 30 minutes before being used. For IP₃ measurements the reactions were stopped at various time points by adding 3.75 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 (IP₁, IP₂, IP₃) were separated as previously described by Berridge et al. Sodium tetraborate (5 mol/L; 32 mL) was added to elute the free inositol and glycerophosphoinositol while IP₁ was eluted from the column with 50 mL of solution containing 0.2 mol/L ammonium formate and 0.1 mol/L formic acid. IP₂ and IP₃ were eluted with 30 mL of 0.4 mol/L ammonium formate–0.1 mol/L formic acid and 1.0 mol/L ammonium formate–0.1 mol/L formic acid solution, respectively. For IP₁, determination, 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 IP₁, had shown that this elution pattern yields 90% recovery.

**Cytosolic ionized Ca²⁺ ([Ca²⁺]) measurement.** Washed platelets were suspended in 1 mL of loading buffer (145 mmol/L NaCl, 10 mmol/L HEPES, 10 mmol/L MgCl₂, 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 CaCl₂ (1 mmol/L) at a concentration of 1 x 10⁹ platelets/mL. [Ca²⁺], was measured in a Deltascan-1 double-beam fluorescence spectrophotometer (Photon Technology International, Bristol, CT) using 1.5-mL aliquots of the platelet suspension. [Ca²⁺], was determined by the calibration procedure of Tsien et al using a kd for fura2 of 224 nm.

**PGI₁, and cGMP radioimmunoassays.** Production of PGI₁ was quantitated by radioimmunoassay of its stable breakdown product 6-keto-PGF₁α 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 10⁹ to 1 x 10¹⁰ cells) for 1 minute causes increasing inhibition of thrombin-induced platelet aggregation (Fig 1A). The anti-aggregatory effect of EC is also observed when platelets are stimulated with collagen (data not shown). The possibility that PGI₁ is involved in the anti-aggregatory effect of EC is excluded because both cell types are pretreated with ASA and 6-keto-PGF₁α, 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-PGF₁α 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 10⁹ cells) for 1 minute increases the cGMP content of platelets from 0.109 ± 0.008 to 0.174 ± 0.006 pmol/10⁶ 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 10⁹ 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 anti-aggregatory 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...
platelet inhibitory effect of EC (1 \times 10^6 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 \textsuperscript{[32P]}orthophosphate-labeled platelets, thrombin (0.1 U/mL) induces a rapid breakdown and resynthesis of PIP\textsubscript{2}, followed by the generation of phosphatidic acid (PA). Incubation of platelets with BAEC (1 \times 10^6 cells) for 1 minute significantly attenuates the breakdown in PIP\textsubscript{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

\begin{figure}[h]
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\caption{Effect of EC on thrombin-stimulated platelet aggregation. Increasing numbers of BAEC (5 \times 10^4 to 1 \times 10^6 cells) produce increasing inhibition of platelet aggregation (A). The platelet inhibitory effect of BAEC (1 \times 10^6 cells) was potentiated by superoxide dismutase (SOD; 60 U/mL), and was prevented by treating platelets with MB (10 \mu mol/L) or by treating BAEC with L-NNA (100 \mu mol/L) (B). The platelet inhibitory effect of BAEC (1 \times 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.}
\end{figure}
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 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 others³⁵,³⁶ 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 not shown), indicating that the inhibition of platelet PA production under these conditions is due to the release of EDRF by EC.

In [³H]myoinositol-labeled platelets, thrombin (0.1 U/mL) stimulates an immediate increase in IP₃ production that peaks 15 seconds after agonist addition and then returns to basal levels within 1 minute. The addition of EC (1 x 10⁶ cells) to the platelet suspension markedly attenuates the thrombin-induced increase in platelet IP₃ levels (Fig 5).
[Ca^{2+}]^{39}$ and to reduce the incorporation of $^{32}$P into myosin light chain$^{39}$ 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$^{30,31}$ and vascular smooth muscle cells.$^{31}$ In addition, lipophilic analogues of cGMP inhibit polyphosphoinositide hydrolysis and Ca$^{2+}$ mobilization in thrombin-stimulated platelets$^{31}$ and in smooth muscle cells.$^{31,41}$ A recent study has shown that nitrovasodilators, which increase platelet cGMP levels,$^{44}$ can also inhibit thrombin-induced platelet PA production.$^{45}$ 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.$^{39}$ 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;$^{46}$ however, this has not been confirmed in platelets.$^{45}$

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$_2$ production has been previously reported$^{42,43}$ 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$_2$ production. Therefore, it appears that both EDRF and PGI$_2$ play an important role in modulating platelet aggregation. The finding that EDRF and PGI$_2$ act synergistically to inhibit platelet aggregation$^{46}$ 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[1] 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.[2] Bradykinin, a stimulus for EDRF release,[3] 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,[4] the lack of release of EDRF from bradylxin-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 PIP2-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.

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W Durante, MH Kroll, PM Vanhoutte and AI Schafer