Activated protein C stimulates the fibrinolytic activity of cultured endothelial cells and decreases antiactivator activity

Yoichi Sakata, Scott Curriden, Dan Lawrence, John H. Griffin, and David J. Loskutoff

Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA 92037

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ABSTRACT The effects of bovine activated protein C (APC) on the fibrinolytic activity of cultured bovine aortic endothelial cells were investigated. Confluent monolayers were incubated with purified APC under various conditions and changes in total fibrinolytic activity and in the level of plasminogen activator and plasminogen activator inhibitor (antiactivator) were monitored. The addition of APC to the cells in the absence of other blood or plasma components led to a rapid, dose-dependent increase of fibrinolytic activity both in the media and in cellular extracts. For example, 3.4 μg of APC per ml resulted in a 15-fold increase of fibrinolytic activity in the medium within 1 hour. The enhanced fibrinolytic activity reflected increases in both the urokinase-related and tissue-type plasminogen activators produced by these cells. Interestingly, treatment of cells with APC also caused a rapid, dose-dependent decrease in antiactivator activity. Diiisopropyl fluorophosphate-inactivated APC did not decrease antiactivator or increase plasminogen activator. Although a small but significant direct (i.e., cell-independent) effect of APC on both fibrinolytic activity and antiactivator activity could be demonstrated, the major portion of these changes appeared to be cell-mediated. These observations indicate that the fibrinolytic potential of cultured endothelial cells is increased by APC and that the enzyme active site is essential for this change. Moreover, the results suggest that one of the primary mechanisms for this stimulation of endothelial cell fibrinolytic activity involves an APC-mediated decrease in antiactivator.

MATERIALS AND METHODS

Reagents. Materials were purchased from the following sources: plasticware from Corning; media from Flow Laboratories; calf serum, trypsin, penicillin, and streptomycin from Gibco; diisopropyl fluorophosphate (iPr2P-F), hirudin (grade IV), bovine serum albumin, Triton X-100, and chloramine-T from Sigma; Tween 80 from Baker; Bio-Rect 70 from Bio-Rad; LFG-agarose from Miles; blue-Sepharose CL-6B and DEAE-Sephadex A-50 from Pharmacia; S-2366 (pyro-Glu-Pro-Arg-paranitroanilide) a gift from Kabo (Möndal, Sweden). All other chemicals were analytical grade.

Proteins. Bovine PC was purified from bovine plasma by using barium citrate adsorption, ammonium sulfate precipitation, and DEAE-Sephadex (16) and blue-Sepharose CL-6B chromatography. Blue-Sepharose CL-6B removed prothrombin from the DEAE-Sephadex fractions containing PC. The purified PC appeared to be at least 95% homogeneous when analyzed on 9% polyacrylamide gels in the presence of NaDodSO4 and was free of detectable factors VII, IX, and X and of prothrombin activity.

Bovine APC was prepared by activation of PC with α-thrombin at an enzyme-to-substrate weight ratio of 1:5. Activation was monitored by the appearance of amidolytic activity toward the chromogenic substrate S-2366. In this assay, 10 μl of APC (0.5–1 mg) was added to 490 μl of 0.05 M Tris-HCl containing 0.13 M NaCl (pH 8.3), 0.1% bovine serum albumin, and 0.15 mM S-2366. Activity was measured at 37°C as the initial ΔA405/min using a Cary 210 spectrophotometer. Thrombin was removed from APC by chromatography on a column of Bio-Rect 70. Although no thrombin activity was detected in the APC as measured in fibrinogen clotting assays, hirudin (0.5 unit/ml final concentration) was added to insure that the preparation was devoid of all thrombin activity. iPr2P-F-inactivated APC was prepared by incubating APC with 5 mM iPr2P-F for 2 hr at 22°C, followed by dialysis. The iPr2P-F-treated sample exhibited no detectable amidolytic activity. Plasminogen and fibrinogen were purified as described (17, 18). 125I-labeled fibrinogen was prepared by using the chloramine-T method (11, 19). Human urokinase (World Health Organization 1st International Reference Preparation) was purchased from the National Institute for Biological Standards and Control (London, U.K.).

Cell Culture. BAECs were isolated from the aortae of freshly slaughtered cows and cultured as indicated (20). The cells used for these studies had been passaged 3–20 times and were positive for factor VIII-related antigen. All cultures were grown to confluency in 60-mm dishes. Confluent cultures were fed every day for 4 days and again 1 hr before use, with modified Eagle’s medium (ME medium) containing 10% calf serum.

Abbreviations: PC, protein C; APC, activated PC; PA, plasminogen activator; u-PA, urokinase-type PA; t-PA, tissue-type PA; iPr2P-F, diisopropyl fluorophosphate; BAEC, bovine aortic endothelial cell.

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Estimated Media (CM) and Cellular Extracts. Confluent cultures were washed three times with phosphate-buffered saline (P/NaCl) (0.01 M sodium phosphate/0.14 M NaCl, pH 7.2) to remove the serum and then incubated at 37°C for various times in serum-free ME medium containing various amounts of APC or iPr2P-F-inactivated APC and hirudin. The CM was collected, centrifuged at 600 × g for 10 min at 22°C to remove detached cells and cellular debris, and stored in 0.01% (vol/vol) Tween 80 at −80°C. To prepare cellular extracts for studies of cell-associated PA, cultures were washed three times with cold (4°C) P/NaCl and then extracted at 4°C with 500 μl of Triton X-100 [0.5% (vol/vol) in P/NaCl]. The culture dish was washed again with 500 μl of P/NaCl, and the extract and wash were pooled and stored at −80°C.

Fibrinolytic Activity. PA activity was assayed on 125I-labeled fibrin-coated multiwell tissue culture dishes (24 wells, 16 mm; Costar, Cambridge, MA) as described (11). The standard cell-free assay contained in 1 ml: 4 μg of human plasminogen, 0.1% gelatin, 0.25% Triton X-100, 0.1 M Tris-HCl, pH 8.1, and a source of PA. Fibrinolytic activity was not observed in the absence of plasminogen and was expressed in relative urokinase international units.

Polyacrylamide Gel Electrophoresis. NaDodSO4/polyacryl-amide slab gel electrophoresis was carried out by using the method of Laemmli (21), employing 10-cm resolving gels containing 9% acrylamide and 2-cm stacking gels of 4% acrylamide. After electrophoresis, portions of the gel containing molecular weight standards were removed and stained with 0.25% Coomassie blue, and the remaining portion of the gel was processed for either fibrin autography or reverse fibrin autography.

Fibrin Autography and Reverse Fibrin Autography. To prepare fibrin agar films, a 2% solution of agarose was mixed with prewarmed (45°C) P/NaCl containing plasminogen and thrombin. Fibrinogen (10 mg/ml) in P/NaCl (37°C) was added, and the solution was mixed and poured onto a glass slide. Final concentrations were 1% agarose, 25 μg of plasminogen per ml, 0.18 unit of thrombin per ml, and 2 mg of fibrinogen per ml. After electrophoresis, the NaDodSO4/polyacrylamide gels to be assayed by fibrin autography were soaked in 2.5% Triton X-100 for 1.5 hr at room temperature, patted dry with a paper towel, and applied to the surface of a fibrin-agar indicator film (13). Fibrin-agar indicator films to be used for reverse fibrin autography were prepared as above but also contained urokinase (0.025 unit/ml) final concentration). The interaction of this urokinase with plasminogen caused the indicator film to lyse. The opaque lysis-resistant zones that develop in the otherwise clear indicator film result from the presence of inhibitors in the NaDodSO4/polyacrylamide gel (14, 22).

Miscellaneous. Protein was determined by the method of Bradford with bovine albumin as a standard (23). 125I was measured by using a Micromed (Horsham, PA) γ spectrometer.

RESULTS

Effect of APC on the Fibrinolytic Activity of BAEs. Washed monolayers were exposed to APC in serum-free ME medium, and at various times thereafter an aliquot of the medium was removed and tested for fibrinolytic activity. The addition of APC led to a rapid, dose-dependent increase of the fibrinolytic activity of CM (Fig. 1). This increase was apparent within the first 15 min and continued for the next 6 hr. iPr2P-F-inactivated APC did not increase the fibrinolytic activity of CM. In these experiments, hirudin was added routinely to the reaction mixtures to neutralize traces of thrombin present in the APC preparation. Controls showed that the APC-dependent increase of fibrinolytic activity was the same in the presence or absence of hirudin and that APC itself had no effect on the activity of purified urokinase, t-PA, or plasminogen (data not shown). No change in the morphology of BAEs incubated in the presence of 3.4 μg of APC per ml was observed over a 24-hr period.

Experiments were performed to determine whether the APC-mediated increase of fibrinolytic activity actually required cells (i.e., cell-dependent effect) or resulted from a direct effect of APC on some component(s) in CM (i.e., cell-independent effect). CM was collected at various times from cells not previously exposed to APC, and floating cells and cellular debris were removed by centrifugation. Each sample then was incubated in vitro for 1 hr at 37°C in the presence or absence of APC and retested for fibrinolytic activity. A small but significant and dose-dependent increase of fibrinolytic activity was observed (Fig. 1). For example, the fibrinolytic activity of 6-hr CM increased by ~2-fold upon incubation with 0.68 μg of APC per ml and by 4- to 5-fold upon incubation in the presence of 3.4 μg of APC per ml. In contrast, when APC was added to the medium in the presence of cells, the fibrinolytic activity continued to rise for at least 6 hr (Fig. 1).

When confluent BAEs were fed with fresh ME medium containing 10% calf serum, incubated for 96 hr without refeeding, and then challenged with APC, increases in fibrinolytic activity similar to those seen in Fig. 1 were observed (data not shown). Thus, although the feeding regimen of BAEs may markedly influence the overall fibrinolytic activi-
Monolayers of BAEs were exposed to various amounts of APC for the indicated times under culture conditions, washed extensively, and extracted with Triton X-100. The fibrinolytic activity of an aliquot of each extract containing 50 μg of protein was measured and expressed as international units (IU) of urokinase.

**Effect of APC on the PAs Produced by BAEs.** Experiments were conducted to determine whether the APC-induced increase in fibrinolytic activity resulted from changes in different forms of PA. CM was collected from cells exposed to APC for 6 hr, subjected to NaDodSO4/polyacrylamide gel electrophoresis, and analyzed by fibrin autography (Fig. 2). CM from confluent BAEs contained multiple molecular forms of PA, seen as distinct lytic zones of Mr 52,000, 74,000, and 100,000 (Fig. 2, lane 1). A weaker activity was observed throughout the region of the gel between the two higher molecular weight forms. APC caused a dose-dependent increase in the activity of all PA forms (Fig. 2, lanes 2 and 3). The PA profile did not change when iPr2P-F-inactivated APC was employed (Fig. 2, lane 4), in agreement with the functional assay data (Fig. 1). No PA activity was detected in purified APC itself (Fig. 2, lane 5).

**Effect of APC on Antiactivator.** BAEs produce both PAs and an antiactivator (13–15). Thus, agents that alter the fibrinolytic activity of these cells may do so by changing PA, antiactivator, or both (24). The effect of APC on the antiactivator activity of BAE CM was studied by reverse fibrin autography (Fig. 3), a semi-quantitative technique in which the size of the lysis-resistant zone in the indicator film reflects the amount of inhibitor applied to the NaDodSO4 gel (22). CM collected from untreated cultures gave rise to a single lysis-resistant zone of Mr 50,000 (Fig. 3, lane 1), reflecting the presence of the antiactivator (22). Considerably less antiactivator activity was detected in CM prepared from APC-treated cells (Fig. 3, lane 2). This decrease in antiactivator was not apparent when iPr2P-F-inactivated APC was employed (Fig. 3, lane 3), consistent with the lack of effect of inactive APC on total fibrinolytic activity (Fig. 1). The APC-mediated decrease in the antiactivator activity of CM was dose-dependent (Fig. 4A). A dose-dependent decrease in cell-associated antiactivator activity also was obvious (Fig. 4B), but to a lesser degree than that observed for CM. Experiments similar to those described in Figs. 1 and 2 were performed to determine whether the decrease in antiactivator resulted from a direct, cell-independent effect of APC on antiactivator or was cell-mediated (Fig. 4C). The pattern in Fig. 4C shows a small but significant effect of APC in the absence of cells (compare Fig. 4C, lanes 1 and 2). However, this cell-independent effect was relatively minor when compared to the decrease in antiactivator observed when APC was added to cells (compare Fig. 4C, lanes 2 and 3).

**Table 1. Effect of APC on cell-associated fibrinolytic activity**

<table>
<thead>
<tr>
<th>Sample</th>
<th>0 hr</th>
<th>1 hr</th>
<th>3 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.5</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>APC</td>
<td>0.68 μg/ml</td>
<td>9.0</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>3.4 μg/ml</td>
<td>25.0</td>
<td>35.0</td>
</tr>
<tr>
<td></td>
<td>3.4 μg/ml, iPr2P-F</td>
<td>2.5</td>
<td>3.0</td>
</tr>
</tbody>
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**Fig. 3.** Analysis of antiactivator released from BAEs exposed to APC. Confluent BAEs were incubated in serum-free medium in the absence of APC (lane 1) or in the presence of either APC (lane 2) or iPr2P-F-inactivated APC (lane 3) at 3.4 μg/ml. Six hours later, the CM was removed and 100 μl of it was analyzed by NaDodSO4/polyacrylamide gel electrophoresis and reverse fibrin autography.

**Fig. 4.** Effect of APC on antiactivator. Confluent BAEs were incubated in serum-free ME medium in the presence of increasing amounts of APC. After 6 hr, 100 μl of the resulting CM (A) or 50 μg of the cell extracts (B) were analyzed for antiactivator activity by reverse fibrin autography. The concentrations of APC employed were 0 (lane 1), 0.68 (lane 2), and 3.4 (lane 3) μg/ml. In separate experiments (C), an aliquot (100 μl) of CM collected from untreated BAEs after 3 hr was incubated for an additional 1 hr in a test tube in the absence (lane 1) or presence (lane 2) of APC (3.4 μg/ml) and similarly analyzed. Lane 3 shows CM collected from cells exposed to APC (3.4 μg/ml) for 3 hr.
DISCUSSION

Bovine APC increases the fibrinolytic activity of blood when it is administered intravenously to dogs (7, 8), possibly through the generation of a secondary messenger molecule that stimulates PA release from endothelium (8). Our results indicate that APC also stimulates the fibrinolytic activity of isolated endothelial cells. However, in apparent contrast to the in vivo results (8), the effect on cultured cells occurs in the absence of plasma or blood cells (Fig. 1), suggesting that the increase in this system is initiated by APC itself and not by a second molecule. The APC effect is dependent on the enzyme active site (Figs. 1 and 2), and both cell-associated and secreted fibrinolytic activity are increased. Interestingly, the APC-mediated stimulation of fibrinolytic activity is achieved both through the direct action of APC on a component(s) in CM and as a consequence of a cellular response to the presence of APC. The direct, cell-independent effect is rapid, dose-dependent, and reproducible. However, its magnitude and duration are relatively small compared to the increases observed in the presence of cells (Fig. 1). Thus, the APC-dependent stimulation of the fibrinolytic system of cultured BAEs appears to be mediated, in large part, by the cells themselves. Whether the responsiveness of the cells to APC is influenced by their growth state (10, 11) and passage number remains to be determined.

The exact mechanisms by which APC increases the fibrinolytic activity of BAEs remain to be elucidated. They are likely to be complex. For example, because BAEs produce both fibrinolytic activators (11–13) and an antiaativator that inhibits them (14, 15), the increase of fibrinolytic activity may be due to increased PA, decreased antiaativator, or both. APC does cause a significant increase in the activity of all PA forms revealed by fibrin autography gels (Fig. 2). However, it also decreases antiaativator activity (Figs. 3 and 4) and does so in a way that is consistent with, and may account for, its effect on total fibrinolytic activity. For example, the APC-mediated decrease in antiaativator in both CM (Fig. 4A) and cells (Fig. 4B) requires the active site of the enzyme (Fig. 3) and occurs in a dose-dependent manner. Moreover, there is both a cell-independent and cell-mediated decrease in antiaativator in response to APC (Fig. 4C). The cell-independent effect of APC on antiaativator, like its direct effect on fibrinolytic activity, is small compared to the cell-mediated changes in these activities. These considerations support the hypothesis that one of the major mechanisms by which APC increases the fibrinolytic activity of BAEs is through these effects on antiaativator. It is not clear whether APC also stimulates the rate of PA synthesis and release or whether the APC-mediated increase of overall fibrinolytic activity (Fig. 1) and of PA activity (Fig. 2) can be accounted for entirely by its effect on antiaativator. APC itself is a serine proteinase (6, 7), and its active site is required both to stimulate fibrinolytic activity (Figs. 1 and 2) and to decrease antiaativator (Fig. 3). However, it is not clear from these experiments whether the direct, cell-independent change(s) is in fact a proteolytic event (e.g., cleavage and inactivation of the antiaativator). APC does not appear to alter directly the apparent molecular weight or fibrinolytic activity of purified t-PA, urokinase, or plasminogen (data not shown), suggesting that if the proteolytic activity of APC is important for the direct effect, it is probably not important at the level of these components. The nature of the cleavage event that initiates the cell-mediated changes also remains to be elucidated. Obviously, many further studies are required to distinguish among these and other possibilities and to establish the exact mechanism by which APC enhances endothelial cell fibrinolytic activity.

It should be noted that Colucci et al. (25) were unable to detect increased fibrinolytic activity in blood after infusion of human APC into spider monkeys. This failure to demonstrate a profibrinolytic property of human APC may reflect inherent differences in the fibrinolytic system of the spider monkey as compared to that of the dog (7, 8) and the cultured bovine endothelial cell. Alternatively, the inability to measure a fibrinolytic response in the spider monkey may indicate that high circulating levels of antiaativator prevent detection of free t-PA in the euglobulin fractions prepared by Colucci et al. (25). A similar complication has been suggested for the failure to detect t-PA in the culture medium of human umbilical vein endothelial cells (24, 26–28).

In conclusion, these results show that APC directly increases the fibrinolytic activity of endothelial cells and further support the hypothesis that APC generated in the vascular system by thrombin bound to thrombomodulin (29) may prevent thrombosis by increasing fibrinolytic activity as well as by inactivating factors Va and VIIia (6, 7). Since deficiencies in PC (1–3), plasminogen (30), and fibrinolytic activity (31) are associated with thrombotic disease, the APC-dependent fibrinolytic activity of endothelial cells may provide a useful model system for studying the regulation of physiologic thrombolysis.

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