Activated Protein C Induces Endothelial Cell Proliferation by Mitogen-Activated Protein Kinase Activation In Vitro and Angiogenesis In Vivo

Mitsuhiro Uchiba, Kenji Okajima, Yuichi Oike, Yasuhiro Ito, Kenji Fukudome, Hirotaka Isobe, Toshio Suda

Abstract—Activated protein C (APC), a natural anticoagulant, has recently been demonstrated to activate the mitogen-activated protein kinase (MAPK) pathway in endothelial cells in vitro. Because the MAPK pathway is implicated in endothelial cell proliferation, it is possible that APC induces endothelial cell proliferation, thereby causing angiogenesis. We examined this possibility in the present study. APC activated the MAPK pathway, increased DNA synthesis, and induced proliferation in cultured human umbilical vein endothelial cells dependent on its serine protease activity. Antibody against the endothelial protein C receptor (EPCR) inhibited these events. Early activation of the MAPK pathway was inhibited by an antibody against protease-activated receptor-1, whereas neither late and complete activation of the MAPK pathway nor endothelial cell proliferation were inhibited by this antibody. APC activated endothelial nitric oxide synthase (eNOS) via phosphatidylinositol 3-kinase-dependent phosphorylation, followed by activation of protein kinase G, suggesting that APC bound to EPCR might activate the endothelial MAPK pathway by a mechanism similar to that of VEGF. APC induced morphogenetic changes resembling tube-like structures of endothelial cells, whereas DIP-APC did not. When applied topically to the mouse cornea, APC clearly induced angiogenesis in wild-type mice, but not in eNOS knockout mice. These in vitro events induced by APC might at least partly explain the angiogenic activity in vivo. This angiogenic activity of APC might contribute to maintain proper microcirculation in addition to its antithrombotic activity. (Circ Res. 2004;95:000-000.)

Key Words: angiogenesis ■ activated protein C ■ endothelial protein C receptor ■ protease-activated receptor-1 ■ mitogen-activated protein kinase

Activated protein C (APC) is a serine protease that plays a central role in physiological anticoagulation. Activation of protein C occurs on the endothelial cell surface by thrombin bound to thrombomodulin. The endothelial protein C receptor (EPCR), which is a glycoprotein on the vascular endothelium, binds to protein C, thereby augmenting protein C activation by the thrombin-thrombomodulin complex. APC regulates the coagulation cascade by inactivating activated forms of factors V and VIII in the presence of protein S, and it exerts a profibrinolytic effect by inactivating plasminogen activator inhibitor inhibitor-1. The importance of APC as a natural anticoagulant is illustrated by the development of severe thrombosis in patients with congenital protein C deficiency.

In addition to the anticoagulant effect, APC has been shown to be capable of activating protease activated receptor (PAR)-1 on endothelial cells, thereby activating the mitogen activated protein kinase (MAPK) pathway. The MAPK pathway is activated during mitosis, meiosis, and G0-G1 transition and is implicated in regulation of the cell cycle, mitogen-induced cell growth, and proliferation. The MAPK pathway plays an important role in signal transduction from growth factor receptors to the nucleus, thereby regulating cell proliferation. These observations strongly suggest that APC might induce endothelial cell proliferation via MAPK activation. Furthermore, because endothelial cell proliferation is a prerequisite step in angiogenesis, it is possible that APC induces angiogenesis.

In the present study, we examined these possibilities using cultured human umbilical endothelial cells (HUVECs) and the mouse cornea angiogenesis assay. Results strongly suggested that APC can induce angiogenesis, probably by activating the MAPK pathway in endothelial cells. Thus, it is likely that APC is a multifunctional protease that might be critically involved in regulation of microcirculation not only by anticoagulation, but by the promotion of neovascularization.
Materials and Methods

Materials

Protein C was purified from human plasma, activated by thrombin, and purified by cation exchange chromatography as described previously. Purified APC displayed a single band on sodium dodecylsulfate polyacrylamide gel electrophoresis. Thrombin activity was not detected in the purified APC concentrate. APC was inactivated by using diisopropyl fluorophosphate (DIP) as described previously. L-Nitro-l-arginine methyl ester (L-NAME) and wortmannin were purchased from Sigma. KT3823, a selective inhibitor of cyclic GMP-dependent protein kinase (PKG), and LY-294,002 were purchased from Alexis corporation. Heat-inactivated supplemented calf serum (SCS) from HyClone Laboratories. Anti-ERK1/2 antibody, anti-phosphorylated ERK1/2 antibody, anti-MEK1/2 antibody, and anti-phosphorylated MEK1/2 antibody were purchased from Cell Signaling. Monoclonal anti-PAR-1 antibody (ATAP2) was purchased from Alexis corporation. Heat-inactivated supplemented calf serum (SCS) containing 10% SCS in 96-well plates. Although cells were grown to confluence at 37°C in humidified 95% air/5% CO2, as described previously, Cells were used for experiments up to passage number 4.

Cell Culture

HUVECs were isolated from fresh human umbilical cord veins as described previously. Cells were grown to confluence at 37°C in humidified 95% air/5% CO2, as described previously. Cells were used for experiments up to passage number 4.

5-Bromodeoxyuridine Incorporation Assay

HUVECs were seeded in 96-well plates at 80% confluence (1×10^4 cells/well) and incubated in M199 with 10% SCS for 24 hours. APC and various pharmacological agents were then added, and after 16 hours, 5-Bromodeoxyuridine (BrdU; 10 μg/mL) was added to each well. Two hours later, the culture media were removed, and the BrdU incorporation was estimated using a BrdU incorporation assay kit (Hoffman La Roche) according to manufacturer’s instructions.

Endothelial Cell Proliferation Assays

Endothelial cell proliferation was monitored by a technique described by Hood et al with some modification. Briefly, HUVECs (5×10^3/well) were incubated with APC and various pharmacological agents in M199 containing 10% SCS in 96-well plates. Although culture media containing 0.2% serum were used in the original assay method, HUVECs in culture media containing 1% serum were demonstrated to be apoptotic at 18 hours after incubation. Thus, we used culture media containing 10% SCS to avoid apoptosis in the present study. After a 4-day incubation, cell number was estimated using a MIT kit (Hoffman La Roche) according to manufacturer’s instructions.

Matrigel Morphogenetic Assay

Formation of endothelial tube-like structures was analyzed using growth factor reduced MATRIGEL matrix (Becton Dickinson). Wells of 48-well culture cluster dishes were coated with 100 μL/well and allowed to solidify for 30 minutes at 37°C. HUVECs, after being starved for 24 hours in M199 with 10% SCS, were seeded on polymerized Matrigel (2×10^4 cells/well) and further propagated for 16 hours, then fixed in PBS containing 3% formaldehyde and 2% sucrose. To quantify the length of tube-like structures, three random phase-contrast photomicrographs (3 objective) per well were taken, and the length of tube-like structures was measured. Tube-like structures were quantified as the average total length of tube-like structures per low-power field.

Measurement of MEK 1/2, Phosphorylated MEK1/2, ERK1/2, Phosphorylated ERK1/2, eNOS, and Phosphorylated eNOS

HUVECs were cultured in 12-well dish. On the day of experiments, culture media was changed M199 with 1% SCS containing various test drugs. After 30-minute incubation period, APC was added to culture media. After indicated time, whole cell lysates were collected and subjected to SDS-PAGE and Western blot analysis as described previously. NIH Image 1.62 was used for densitometric quantification of the bands.

Measurement of NO2/NO3

Confluent HUVECs were treated with M199 plus 10% SCS in the presence and absence of APC for 1 hour at 37°C. Medium was collected and NO2/NO3 levels in culture media were measured using a nitric oxide colorimetric assay kit (Hoffman La Roche).

Measurement of cGMP Levels in HUVECs

Confluent HUVECs were treated with M199 plus 10% SCS in the presence and absence of APC for 30 minutes at 37°C. After incubation, cells were washed by ice-cold PBS and incubated with ice-cold 65% ethanol. Ethanol solution was collected to new tube, dried under nitrogen, and stored till cGMP measurement. Levels of cGMP were performed according to protocols supplied by the manufacturer (Amersham Pharmacia Biotech).

Mouse Corneal Angiogenesis Assay

The mouse corneal assay was performed according to procedures described. Under sterile conditions, slow-release pellets were prepared incorporating APC or various material into a casting solution of a ethylvinyl copolymer (Elvax-40, DuPont), in 10% methylene chloride. Male 8-week-old C57BL/6 mice or eNOS knockout mice were anesthetized with sodium pentobarbital, corneal micropockets were created in the corneal stroma, and pellets were implanted. The eyes were examined by a slit-lamp biomicroscope (Kowa Company, Ltd., Nagoya, Japan) on day 6 after pellet implantation. Angiogenic responses were evaluated by vessel length and neovascularization area of cornea.

Statistical Analysis

Statistical analysis was performed with analysis of variance followed by Scheffe’s post hoc test for multiple comparison. A level of P<0.05 was used as statistically significant.

Results

Effects of APC and DIP-APC on MAPK Pathway Activation in Endothelial Cells

To confirm that APC activates the MAPK pathway in endothelial cells, we examined the effect of APC on phosphorylation of MEK1/2 and ERK1/2 in cultured HUVECs in the present study. As shown in Figure 1A, intracellular levels of phosphorylated MEK1/2 began to increase 5 minutes after treatment with APC (300 nmol/L), peaking at 15 minutes after treatment, and thereafter gradually decreased to pretreatment levels. Intracellular levels of phosphorylated ERK1/2 began to increase from 5 minutes after treatment with APC, peaking at 15 minutes after the treatment, and thereafter gradually decreased to pretreatment levels (Figure 1A). To determine whether serine protease activity of APC is critical for APC-induced MAPK activation in endothelial cells, we examined the effect of DIP-APC, an active site-blocked APC, on the phosphorylation of ERK1/2 in cultured HUVECs. As shown in Figure 1B, DIP-APC did not increase intracellular levels of phosphorylated ERK1/2.

Effects of APC and DIP-APC on BrdU Incorporation and Proliferation of Endothelial Cells

APC significantly increased BrdU incorporation and proliferation of endothelial cells in a concentration-dependent
manner: half-maximal concentrations of APC for BrdU incorporation and proliferation of endothelial cells were 6.5 and 10 nmol/L, respectively. (Figure 1B and 1C). DIP-APC (300 nmol/L) did not increase BrdU incorporation and proliferation of endothelial cells (Figure 1B and 1C), suggesting that serine protease activity of APC might be essential for the induction of DNA synthesis and proliferation of endothelial cells.

Effects of Anti-EPCR Antibodies and an Anti–PAR-1 Antibody on Activation of the MAPK Pathway, DNA Synthesis, and Proliferation Seen in Endothelial Cells Treated With APC

As shown in Figure 2A, anti-EPCR antibody 252, which inhibits the interaction of APC with EPCR,17 significantly reduced APC-induced MAPK activation observed 15 minutes after activation in cultured HUVECs. In contrast, anti-EPCR antibody 92, which does not affect the binding of APC to EPCR,17 did not have any effect (Figure 2A). Increase in BrdU incorporation and proliferation of endothelial cells induced by APC were significantly inhibited by anti-EPCR antibody 252, but not by anti-EPCR antibody 92 (Figure 2B and 2C).

To determine whether PAR-1 plays an important role in APC-induced endothelial MAPK activation and endothelial cell proliferation, we examined the effect of anti-PAR-1 antibody on APC-induced events in cultured HUVECs. Anti-PAR-1 antibody used in the present study completely inhib-
ited endothelial MAPK activation, increase in BrdU incorporation, and proliferation of endothelial cells induced by thrombin (Figure 3). However, although the antibody inhibited endothelial MAPK activation at 5 minutes after treatment with APC (Figure 3A), it did not inhibit MAPK activation at 15 minutes after treatment with APC (Figure 3B). Neither increase in BrdU incorporation nor proliferation of endothelial cells induced by APC was inhibited by anti-PAR-1 antibody (Figure 3C and 3D). These observations suggested that PAR-1 might be implicated in the early activation of MAPK, but might not be critically involved in the late and complete activation of MAPK, leading to DNA synthesis and endothelial proliferation, in cultured HUVECs treated with APC.

Effect of APC on Nitric Oxide Production and Phosphorylation of eNOS in Endothelial Cells

To examine whether APC increases the endothelial nitric oxide (NO) production, we measured NO$_2$/NO$_3$ concentrations in culture media of HUVECs treated with APC. Levels of NO$_2$/NO$_3$ in culture media of HUVECs at 1 hour after addition of APC (300 nmol/L) (10.0±1.9 μmol/L) were significantly higher than those at 1 hour after addition of saline (6.7±1.3 μmol/L, P<0.01).

To determine whether APC might activate eNOS, thereby increasing NO production in endothelial cells, we examined the effect of APC on phosphorylated eNOS levels in endothelial cells. As shown in Figure 4A, APC increased phosphorylation of eNOS. APC-induced phosphorylation of eNOS was inhibited by LY-294,002 and wortmannin, both of which are inhibitors of PI3-kinase (Figure 4B).

Effects of L-NAME, LY-294,002, and Wortmannin on MAPK Activation, Increase in BrdU Incorporation, and Proliferation Seen in Endothelial Cells Treated With APC

To determine whether NO plays a role in APC-induced MAPK activation and endothelial cell proliferation, we examined the effect of L-NAME, an inhibitor of NOS, on endothelial MAPK activation, DNA synthesis, and proliferation seen in endothelial cells treated with APC. As shown in Figure 5, L-NAME inhibited these changes induced by APC. Furthermore, LY-294,002 and wortmannin also inhibited MAPK activation, increase in BrdU incorporation, and proliferation seen in endothelial cells treated with APC.

These observations strongly suggested that APC might activate eNOS via PI3-kinase–dependent phosphorylation, thereby increasing NO production, and the NO thus formed might play a critical role in the endothelial MAPK activation, DNA synthesis, and proliferation seen in endothelial cells treated with APC.

Effect of APC on Intracellular Levels of cGMP and Effect of KT5823, a PKG Inhibitor, on the Endothelial MAPK Activation, Increase in BrdU Incorporation, and Proliferation Seen in Endothelial Cells Treated With APC

Intracellular levels of cGMP in endothelial cells were increased by APC treatment (1.78±0.65 fmol/well in control...
versus 3.13±0.83 fmol/well in APC treatment; \( P<0.01 \). KT5823, an inhibitor of PKG, inhibited MAPK activation, increase in BrdU incorporation and proliferation seen in endothelial cells treated with APC (Figure 5). These observations strongly suggested that APC might activate the MAPK pathway, thereby inducing DNA synthesis, endothelial cell proliferation, and MAPK activation could be induced by PKG, which was activated via an NO-dependent increase in intracellular levels of cGMP in endothelial cells treated with APC.

**APC Induces Morphological Differentiation**

To examine whether APC induces morphogenetic changes resembling tube-like structures, we investigated the effect of APC on the morphological change of HUVECs plated on Matrigel. Treatment with APC for 16 hours resulted in morphological changes, including elongation and formation of thin cords of interconnecting cells (Figure 6B and 6D). These changes were not induced by DIP-APC treatment (Figure 6C and 6D).

**Effect of APC on Angiogenesis In Vivo**

Because APC induced the endothelial cell proliferation by activating the MAPK pathway in cultured HUVECs as shown in the present study, it is possible that APC induces angiogenesis in vivo. We examined this possibility using the mouse corneal angiogenesis model. As shown in Figure 7C, APC induced corneal angiogenic response; ie, new microvessels crossed the cornea from the limbus toward the pellet containing APC. Angiogenic response was not observed with the pellet alone (Figure 7A). In this experimental condition, APC-induced corneal angiogenic response was comparable to that of VEGF (Figure 7B). DIP-PAC, active site blocked APC, did not show any angiogenic response (Figure 7D). APC-induced corneal angiogenic response was not observed in eNOS knockout mice (Figure 7F).

**Discussion**

In the present study, we demonstrated that APC induced activation of both MEK1/2 and ERK1/2, increase in DNA...
synthesis, and proliferation in cultured HUVECs. Because activation of this MAPK system has been shown to be a prerequisite for growth factor–stimulated mitogenesis in many cells, endothelial cell proliferation induced by APC might be at least partly explained by activation of these enzymes.

Phosphorylation of eNOS was increased by APC, which was inhibited by LY-294,002 and wortmannin. Furthermore, L-NAME, LY-294,002, and wortmannin inhibited both MAPK activation and endothelial cell proliferation induced by APC. Nitric oxide emerged as an important upstream signal of cell proliferation in endothelial cells. Hood et al reported that nitric oxide plays critical roles in the signaling pathways and endothelial cell proliferation induced by VEGF. These observations strongly suggested that APC might activate eNOS via PI3-kinase–dependent phosphorylation, thereby inducing both MAPK activation and endothelial cell proliferation.

The precise mechanism(s) by which APC signals in cultured HUVECs, thereby activating the MAPK pathway, is not fully elucidated. Recently, Riewald et al reported that APC activates PAR-1 by interacting with EPCR on endothelial cells. They also showed that EPCR-bound APC induced phosphorylation of MAPK via PAR-1 activation. These observations are consistent with our present observations that endothelial MAPK activation was inhibited by anti-EPCR antibody 252 that is capable of inhibiting the interaction of APC with EPCR and that the activation was not induced by DIP-APC, an inactive derivative of APC. Riewald et al also demonstrated that anti–PAR-1 antibody significantly inhibited MAPK activation induced by APC observed at 6 minutes after treatment with APC. Consistent with this observation, anti–PAR-1 antibody significantly inhibited APC-induced MAPK activation in cultured HUVECs 5 minutes after treatment with APC as shown in the present study. However, anti–PAR-1 antibody inhibited neither MAPK activation at 15 minutes after treatment of endothelial cells with APC, increase in DNA synthesis, nor proliferation in cultured HUVECs treated with APC. Because this antibody completely inhibited both MAPK activation and endothelial cell proliferation induced by thrombin as shown in the present study, PAR-1–mediated MAPK activation and the subsequent endothelial cell proliferation could be completely inhibited by the antibody in the present experimental conditions. These observations suggested that PAR-1 might be implicated in early MAPK activation, but might not be critically involved in the late and complete activation of MAPK leading to endothelial proliferation in cultured HUVECs treated with APC. Further studies are necessary to determine the mechanism(s) by which APC signals the endothelial cells to activate the MAPK pathway.

Recently, Domotor et al reported that EPCR-bound APC induced intracellular calcium mobilization from the endoplasmic reticulum in cultured human umbilical vein endothelial cells. Because ionomycin-induced calcium release activates the ERK1/2 in cultured HUVECs and thrombin-induced MAPK activation was inhibited by the intracelular calcium chelator, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM), it is possible that APC activates the MAPK pathway by inducing intracellular calcium mobilization. However, this possibility seems less likely because APC-induced intracellular calcium mobilization was shown to be a PAR-1–dependent process. Furthermore, observations in our preliminary experiments demonstrated that APC-induced MAPK activation was only partially inhibited by BAPTA-AM, whereas thrombin-induced MAPK activation was almost completely inhibited by BAPTA-AM, suggesting that APC-induced MAPK activation might not be mediated by calcium mobilization in cultured HUVECs in the present study.

In normal human subjects, plasma levels of protein C and APC are reported to be 65 nmol/L and 38 pmol/L, respectively. Because APC is formed by thrombin bound to thrombomodulin on the endothelial surface, local concentration of APC might be increased at the site where thrombin generation is increased. Consistent with this hypothesis, Hanson et al reported that plasma levels of APC were found to be 8 nmol/L in baboons given thrombin intravenously.
mean SD of three in each group. Typical results are shown for

Figure 7. Effects of APC, DIP-APC, and VEGF on angiogenesis
in vivo. Pellets containing control buffer (A and E), VEGF (B),
APC (C and F), or DIP-APC (D) were implanted into corneal
micropockets of C57BL/6 mice (A-D) or eNOS knockout mice (E
and F). Corneas were photographed with a slit-lamp biomicro-
scope on day 6 after pellet implantation. Maximal vessel length
(G) and area of neovascularization (H) are presented as
mean±SD of three in each group. Typical results are shown for
three animals examined in each group. *P<0.01 vs Control of
wild type, †P<0.01 vs APC of wild type.

Because 8 nmol/L was a half-maximal concentration of APC
required for the induction of increase in DNA synthesis and
endothelial cell proliferation as shown in the present study, it
is possible that APC induces endothelial cell proliferation
leading to angiogenesis at the site where thrombin generation
is increased.

Physiological relevance of angiogenesis induced by APC is
not clear at present. Development of the vascular system in
various organs in protein C knockout mice was reported to be
normal during embryogenesis.23 EPCR knockout mice showed early embryonic lethality, but normal vascular system
development.24 These results suggested that APC might not be
involved in vasculogenesis and embryonic angiogenesis.
Postneonatal angiogenesis, referred to as neovascularization,
was observed in various physiological and pathological
conditions such as wound healing, postischemic tissue resto-
ration, and carcinogenesis.25 Expression of EPCR was dem-
onstrated to be enhanced in carcinomatous tissues of breast
cancer, melanomas, and renal and colon carcinomas,26,27
suggesting that APC might be critically involved in neovas-
cularization in these carcinomatous tissues. Neovasculariza-
tion is also observed in ischemic tissues.28 Hypoxia activates
the coagulation cascade by increasing the endothelial expres-
sion of tissue factor, a triggering substance for the coagula-
tion cascade, leading to thrombin generation.29 Thrombin
thus generated binds to thrombomodulin, thereby activating
protein C to form APC,3 suggesting that generation of APC
might be increased in the ischemic tissue. APC was shown to
be beneficial in reducing ischemia/reperfusion-induced injury
of the kidney,30 spinal cord,31 and brain17 based on its
antiinflammatory properties. In addition, such therapeutic
effects of APC might be at least partly explained by angiogenic
activity, leading to the neovascularization by which
ischemic tissue injury can be attenuated through restoration
of the blood supply. This possibility should be further
investigated using protein C knockout mice subjected to the
ischemia-induced tissue injury in the near future.

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Measurement of thrombin activity in APC concentrates

Thrombin activity was measured amidolytically using the chromogenic substrate S-2238 (Chromogenix AB, Stockholm, Sweden) in APC concentrates as described previously with some modification (1). In brief, APC concentrates were diluted with buffer containing 50 mmol/L Tris–HCl, (pH 7.4), 150 mmol/L NaCl, 5 mmol/L CaCl₂, and S-2238 was added at a final concentration of 0.4 mmol/L. The increase in the absorbance at 405 nm was determined for 5 minutes at 37 °C. Since APC has amidolytic activity towards S-2238, we determined effects of argatroban (1 μmol/L), a selective inhibitor of thrombin, or heparin (1U/mL) plus antithrombin (500 nmol/L) on the amidolytic activity seen in APC concentrates. To confirm the inhibition of thrombin activity by these agents, we also determined the amidolytic activity in solution containing purified thrombin (5 U/ml) (Mitsubishi-welpharma Co, Osaka, Japan) instead of APC. Antithrombin plus heparin and argatroban at these concentrations significantly inhibited the increase in the absorbance at 405 nm in thrombin solution, while they did not inhibit it in APC concentrates as shown in Table 1. These results indicated that thrombin was not contaminated in APC concentrates used in the present study.
Reference

Table 1. Effects of argatroban and antithrombin plus heparin on amidolytic activity of APC concentrate and purified thrombin concentrate.

<table>
<thead>
<tr>
<th>Solution</th>
<th>A405 (DOD/min)</th>
</tr>
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<tbody>
<tr>
<td>APC (3 µmol/L)</td>
<td>0.470 ± 0.017</td>
</tr>
<tr>
<td>with heparin plus antithrombin</td>
<td>0.469 ± 0.021</td>
</tr>
<tr>
<td>with argatroban</td>
<td>0.479 ± 0.019</td>
</tr>
<tr>
<td>Thrombin (5U/mL)</td>
<td>0.400 ± 0.012</td>
</tr>
<tr>
<td>with heparin plus antithrombin</td>
<td>0.002 ± 0.001 *</td>
</tr>
<tr>
<td>with argatroban</td>
<td>0.045 ± 0.004 *</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD of three experiments. Amidolytic activity was determined by measuring hydrolysis of S-2238 as described in supplement. *, p< 0.01 vs. Thrombin (ANOVA followed by Scheffe's post hoc test).