In vivo and In Vitro Complexes of Activated Protein C With Two Inhibitors in Baboons

By Francisco España, András Gruber, Mary J. Heeb, Stephen R. Hanson, Laurence A. Harker, and John H. Griffin

In vivo complex formation of activated protein C with protein C inhibitor (APC-PCI) and with α1-antritrypsin (APC-α1,AT) following infusion of 0.25 or 1.0 mg APC/kg in 1 hour into baboons was studied using immunoblotting and sandwich enzyme-linked immunosorbent assay (ELISAs). Before APC infusion, detectable plasma levels (about 30 ng/mL) of APC-α1,AT complex were found in the baboon plasma. At the lower APC dose, APC-PCI and APC-α1,AT complex levels were 1.4 ± 0.5 (mean ± SE) and 0.8 ± 0.1 μg/mL after 1 hour of infusion. At the higher APC dose, the APC-PCI level was similar to the APC-α1,AT level during the first 30 minutes, but after 1 hour of infusion the APC-α1,AT level was higher than the APC-PCI level, reaching 4.1 ± 1.2 and 2.9 ± 1.2 μg/mL, respectively. After 24 hours, complex levels had returned to basal conditions. During infusion of protein C (1.0 mg/kg in 1 hour), both complexes were detected in low concentrations. Following bolus injection of APC, half-lives (t1/2) for APC and APC-PCI and APC-α1,AT complexes of 10, 40, and 140 minutes, respectively, were observed. After 1-hour incubation with 2.5 μg/mL APC, baboon plasma contained 1.0 ± 0.2 and 0.8 ± 0.1 μg/mL of APC-PCI and APC-α1,AT, respectively. Addition of 10 μg/mL APC to baboon plasma yielded 2.5 and 2.4 μg/mL APC-PCI and APC-α1,AT after 1 hour, respectively. Immunoblotting analysis also showed in vivo formation of complexes of APC with an auxiliary inhibitor but not in vitro in citrated plasma. These data show that both PCI and α1,AT are physiologic inhibitors of APC and suggest that when PCI is depleted by a high dose of APC, α1,AT becomes the major inhibitor of APC. © 1991 by The American Society of Hematology.

MATERIALS AND METHODS

Blood collection. Baboons bearing chronic A-V shunts as described elsewhere21 were used for these studies. Baboon arterial blood samples were collected from the external iliacal shunt in 0.13 mol/L sodium citrate containing 0.3 mol/L benzamidine (nine parts of blood, one part anticoagulant) before, during, and after distal infusion of APC into the external shunt. Pooled normal human plasma (NHP) was made from 25 apparently healthy male donors. Protein C-depleted plasma was from George King Biomedical, Overland Park, KS. Rabbit antisemur to α1,AT (IgG fraction) and horse radish peroxidase were from Sigma, St Louis, MO.

Preparation of proteins and antibodies. Activated protein C,21 protein C inhibitor (PCI),12 and purified APC-PCI21 and APC-α1,AT complexes21 were obtained as indicated. Anti-light chain monoclonal antibody to protein C (C3) was prepared and characterized as described.21,22,24 This antibody is specific for the light chain of protein C, and recognizes protein C, APC, and complexed APC with the same efficiency.16,17 PCI-depleted plasma21,24 and rabbit anti-PCI specific antibodies (IgG fraction) labeled with horse radish peroxidase21,24 were prepared as previously reported.

Assays. APC plasma levels were measured as previously described.21 PCI antigen,22 APC-PCI,22 and APC-α1,AT22 complexes were determined as indicated. Briefly, PCI antigen was assayed in microtiter plates coated with rabbit anti-PCI antibodies and PCI bound was detected with peroxidase labeled anti-PCI antibodies. The detection limit was 0.5 ng/mL for plasma PCI and the assay detected equally well free PCI and complexed PCI. APC complexes were assayed by coating plates with C5 monoclonal anti-protein C antibody, and bound APC complexes were detected with peroxidase-labeled rabbit anti-PCI or anti-α1,AT antibodies. The detection limit for APC-PCI and APC-α1,AT assays was 5 ng/mL and 0.5 ng/mL, respectively. Calibration curves were constructed using known amounts of purified preformed APC complexes.22

To study complex formation in vitro, 5 μL of purified human APC (2.5 or 10.0 μg/mL, final concentration) in 0.01 mol/L TRIS-HCl, 0.14 mol/L NaCl, pH 7.4 (TBS) containing 1% bovine serum albumin (BSA) and 50 μg/mL soybean trypsin inhibitor (STI) was added to 100 μL baboon plasma, in the presence or absence of protein C into animals as well as after in vitro addition of APC to baboon plasma, in the presence and in the absence of heparin. The half-lives of APC and APC complexes in the circulation were determined after bolus injection of APC into baboons.

From the Committee on Vascular Biology and Department of Molecular and Experimental Medicine, Scripps Clinic and Research Foundation, La Jolla, CA.

Submitted September 24, 1990; accepted December 18, 1990.

Supported in part by grants from the National Institutes of Health (HL-31950), the Califomia Affiliate of the American Heart Association (A.G.), and the Fondo de Investigaciones Sanitarias de la Seguridad Social (9010586), Spain.

Address reprint requests to John H. Griffin, PhD, Department of Molecular and Experimental Medicine, BCRS, Research Institute of Scripps Clinic, 10666 N Torrey Pines Rd, La Jolla, CA 92037.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1991 by The American Society of Hematology.

0006-4971/91/7708-0012$3.00/0


1754
absence of different heparin concentrations. As a control, plasma was incubated with TBS-BSA-STI alone. At different time intervals, aliquots were withdrawn and diluted with blocking buffer-benzamidine (TBS containing 1% casein, 30 mmol/L benzamidine, 0.05% Tween 20, 0.05% sodium azide, pH 7.4), and APC-PCI and APC-\(\alpha\)-AT were measured as indicated above. For comparison, APC (2.5, 5.0, or 10 \(\mu\)g/mL) was incubated with a mixture of purified PCI (4.5 \(\mu\)g/mL) and \(\alpha\)-AT (2.5 mg/mL) at the final concentrations indicated. At different times, aliquots were removed and, after dilution with blocking buffer-benzamidine, APC complexes formed were measured as indicated above.

For the in vivo complex formation experiments, citrated baboon plasma samples were analyzed following infusion of 0.25 or 1.0 mg APC/kg in 1 hour into five different animals (30% as initial bolus; remainder by continuous infusion). For these studies, each baboon was fitted with a thrombogenic Dacron vascular graft inserted in a silicone rubber shunt between the femoral artery and vein. In control studies, the same baboons were infused with 1.0 mg protein C/kg in 1 hour. Blood samples were taken into citrate containing 0.3 mol/L benzamidine at different time intervals before, during, and after infusion of APC or protein C, and APC complexes were measured in the derived plasma as indicated above.

To determine the half-life for APC and its complexes in the circulation in the absence of a thrombogenic Dacron graft, two baboons (each 12 kg weight) were injected with 2.5 mg human APC as an intravenous bolus into the silastic A-V shunt. Blood samples were taken into citrate containing 0.3 mol/L benzamidine at different time intervals and analyzed for APC activity and APC complexes, as indicated above.

**Immunoblotting.** Immunoblotting of baboon plasma samples was performed as previously described for human plasma. Briefly, 5% non-denaturing gels were used for separation of protein C, PCI, and APC complexes. After transfer of proteins to nitrocellulose paper, immunoblots were reacted either with antilight chain monoclonal antibody to protein C (C3) at 30 pg/mL, followed by \(\text{\textsuperscript{125}}\text{I}\)-protein C, or with the IgG fraction of rabbit antibody to PCI at 20 \(\mu\)g/mL, followed by \(\text{\textsuperscript{125}}\text{I}\)-PCI. The specific radioactivity of these proteins ranged from 15 to 27 \(\mu\)Ci/\(\mu\)g, and they were diluted to 0.3 \(\mu\)Ci/mL for immunoblotting.

**RESULTS**

**Complexes of activated protein C in vivo.** To study enzyme-inhibitor complex formation in vivo, baboon plasma samples were analyzed following infusion of 0.25 (low dose) or 1.0 (high dose) mg APC/kg during 1 hour. Figure 1 shows the mean values obtained from five different experiments using five animals. Before APC infusion, no APC-PCI complexes were detected, but low levels of circulating APC-\(\alpha\)-AT complexes (about 30 ng/mL) were. During infusion, complexes of APC were detectably formed and increased in concentration with time. At the low APC dose, APC-PCI and APC-\(\alpha\)-AT complex levels were 1.4 \(\pm\) 0.3 and 0.8 \(\pm\) 0.1 \(\mu\)g/mL, respectively, after 1 hour of infusion (Fig 1A). At the higher APC dose used, both complexes showed similar levels during the first 30 minutes, but at 1 hour they were 2.9 \(\pm\) 1.2 and 4.1 \(\pm\) 1.2 \(\mu\)g/mL, respectively (Fig 1B). At the high APC dose, APC-PCI concentration decreased as soon as the infusion of APC finished, whereas the concentration of APC-\(\alpha\)-AT complex continued to increase for 15 minutes after infusion (Fig 1B). Twenty-four hours after APC infusion there was a detectable level of circulating APC-\(\alpha\)-AT complex, similar to that found in basal conditions, but no APC-PCI complex was detected. During a protein C zymogen infusion in baboons fitted with the thrombogenic Dacron shunt, low but detectable levels (between 150 and 200 ng/mL) of both APC-inhibitor complexes were measured (data not shown).

Studies were performed to correlate the appearance of APC complexes measured by enzyme-linked immunosorbent assay (ELISA) to their appearance on immunoblots. Figure 2 shows an immunoblot for protein C antigen in plasma samples from a baboon infused with a low dose of APC. Before APC infusion (1 = 0 minutes), low levels of the band for APC-\(\alpha\)-AT complexes, but not of the band for APC-PCI complexes, were observed. After 5 minutes of infusion, the band containing APC-\(\alpha\)-AT complexes increased above the basal level, and the band for APC-PCI complexes appeared. Bands containing each type of complexes increased in intensity during the 60-minute infusion, then decreased at later time points. By 120 minutes the band of APC-PCI complexes in all experiments was less intense than the band of APC-\(\alpha\)-AT complexes. A third band of low electrophoretic mobility containing APC antigen (see upper region of gel in Fig 2) appeared after 5 minutes of APC infusion, and this band was less intense than the bands for either the APC-PCI or the APC-\(\alpha\)-AT complexes. It also increased during the infusion, then decreased to barely detectable levels by 120 minutes. Bands denoted APC-PCI and APC-\(\alpha\)-AT in Fig 2 comigrated with the major bands of complexes formed when citrated plasma was incubated in vitro with APC or with a snake venom activator of protein C (data not shown). These bands were also identified by coblotting with antibodies to PCI and to \(\alpha\)-AT (Fig 3 and data not shown). The band of low mobility was identified as APC-\(\alpha\)-macroglobulin by its removal using monoclonal antibody to \(\alpha\)-macroglobulin and protein A-Sepharose. The counterpart to this band in citrated human or baboon plasma incubated with APC was very faint or not detectable.

Figure 3 shows an immunoblot for PCI antigen in plasma
of a baboon infused with a high dose of APC. During APC infusion, a new band corresponding to the APC-PCI complex was formed that comigrated with a band observed on immunoblots for protein C antigen. This band remained detectable 1 hour after the infusion was stopped (120 minutes). At the time the infusion was terminated (1 hour), most of the PCI antigen was in the form of APC-PCI complexes. An apparent decrease in free PCI antigen was clearly seen after 15 minutes of infusion (Fig 3).

**In vivo half-life for APC activity and APC-inhibitor complexes.** To study the clearance rate of APC and its complexes with PCI and α1AT in plasma, 2.5 mg APC was injected into two baboons and blood samples taken at time intervals and analyzed for APC amidolytic activity and the two respective APC-inhibitor complexes. After APC infusion, the free active enzyme reached a peak value of 1.0 to 1.6 μg/mL in 2 or 3 minutes, then decreased as an exponential function of time, with a half-life calculated of...
IN VIVO INHIBITION OF ACTIVATED PROTEIN C

To determine if some plasma component could influence the rate of APC complex formation with PCI and α,AT, we studied the time course of APC complexation with a mixture of purified PCI and α,AT (Fig 7) at their respective physiologic plasma concentrations. No differences were seen when these results using purified proteins were compared with those in which APC was incubated with citrated baboon plasma (Fig 5). Therefore, the rate of reaction of APC with PCI and α,AT is not significantly influenced by other plasma components.

DISCUSSION

During infusion into baboons, APC associates with two major inhibitors, PCI and α,AT, to give stable complexes of different electrophoretic mobilities on non-denaturing gels (Fig 2), and these complexes could be quantitated by specific sandwich ELISAs. Apparent complexes of APC with an auxiliary inhibitor were observed on immunoblots, and these complexes were identified as APC-α,macroglobulin.24 We have recently reported APC complexes of similar low mobility on immunoblots of neat human blood but not citrated plasma incubated with APC, and these were identified as APC-α,macroglobulin.25 In another study, APC-PCI, APC-α,AT, and complexes of low electrophoretic mobility were reported in plasma of chimpanzees infused with phospholipid vesicles containing factor Xa.26

Fig 4. Clearance rate of APC and APC complexes in baboons. Human APC (2.5 mg) was administered to two animals. Blood samples were withdrawn at different time intervals into citrate-benzamidine and assayed for APC amidolytic activity (A), and APC-PCI (B), and APC-α,AT (C) complexes, as indicated in Materials and Methods. The figures obtained with the two different baboons are indicated by closed and open circles.
Baboon plasma was incubated at 37°C in the presence or absence of heparin with APC (A, 2.5 μg/mL; B, 10 μg/mL) in TBS containing 1% BSA and 50 μg/mL STI. At time intervals, 10 μL of the incubation mixture was diluted 1:40, 1:80, and 1:160 with blocking buffer containing 30 mmol/L benzamidine and APC complexes were measured as indicated in Materials and Methods. The results are the mean of three different experiments. (●—●) APC-PCI. (○—○) APC-α,AT.

However, the low mobility complexes were thought by the authors not to be APC-α,-macroglobulin.

At the lower APC dose used (0.25 mg/kg/hour), approximately 64% of the complexes detected by ELISAs were APC-PCI and 35% APC-α,AT (Fig 1). These values contrast with those obtained in in vitro studies, where addition of 2.5 or 10 μg/mL (final concentration) of APC to baboon plasma resulted in a similar appearance rate of both complexes (Fig 5). Furthermore, injection of APC into baboons as a bolus showed that, after reaching a peak, APC complexes fell showing a half-life of 40 minutes for APC-PCI, 3 times lower than the 140-minute half-life for APC-α,AT (Fig 4B and C). Despite the shorter half-life for APC-PCI as compared with APC-α,AT complexes, the observed level of APC-PCI complex during APC infusion was nearly 2-fold that of APC-α,AT complex, suggesting in vivo stimulation of PCI complexation with and inhibition of APC, similar to the manner in which heparin increased the in vitro formation of APC-PCI complexes.

Infusion of 1.0 mg APC/kg/h into baboons resulted in APC-α,AT complex levels higher than the APC-PCI complex levels, probably because of depletion of PCI by an excess of APC.

The fact that the two APC complexes in plasma arise at approximately the same rate in the absence of heparin, in vitro, is in agreement with the data reported by Heeb et al. and is consistent with the kinetic constants for the APC inhibition by human PCI and α,AT. España et al. reported a second-order rate constant of 0.65 × 10^-4 mol/L^-1 second^-1 for APC inhibition by PCI in the absence of heparin and hence have reported a value of 10 mol/L^-1 second^-1 for the APC inhibition by α,AT. Although the latter value is 600 times lower than that for APC inhibition by PCI, this difference is inversely proportional to the difference in plasma concentrations of these APC inhibitors. PCI plasma concentration is 5 μg/mL, whereas α,AT concentration is about 3,000 μg/mL, ie, a factor of 600.

APC may play a role as a major regulator of thrombosis because homozygous protein C deficient infants suffer potentially fatal thrombotic disease and hereditary thrombophilia is associated with heterozygous protein C deficiency in some families. In one study, heterozygous protein C deficiency appeared in 6 of 141 patients under 45 years of age with deep vein thrombosis. Moreover, APC inhibits platelet-dependent arterial thrombosis in a baboon model and prevents the coagulopathy as well as the fatal outcome associated with Escherichia coli-induced shock in a baboon model of septicemia. Therefore, the regulation of APC anticoagulant activity in blood by its inhibitors, PCI and α,AT, is of major physiologic importance. Previous studies have demonstrated the presence of circulating APC complexed to PCI and to α,AT in various diseases.
IN VIVO INHIBITION OF ACTIVATED PROTEIN C

states such as deep venous thrombosis, cerebral arterial thrombosis, and disseminated intravascular coagulation. However, the majority of patients studied showed APC-α,AT complex levels much higher than APC-PCI complex levels, which is in contrast to the results found here for APC infusions. APC-PCI is probably cleared from the human circulation more rapidly than APC-α,AT complex as seen here, and this difference in APC-inhibitor half-life would explain the lower APC-PCI levels detected in patients. We show here that APC-PCI complexes and APC-α,macroglobulin complexes are cleared from the baboon circulation 3 times faster than are APC-α,AT complexes (Fig 4B and C). Six hours after APC infusion into baboons there were significant residual amounts of APC-α,AT complexes (about 100 ng/mL), whereas the level of APC-PCI was lower than 10 ng/mL by that time. It is not known yet which other enzymes may compete for PCI in different disease states. Plasma kallikrein, factor XIa, and thrombin also can complex to and cleave PCI. These enzymes are generated during thrombosis or consumptive coagulopathy and may abolish the inhibitory activity of PCI, thereby making α,AT the major inhibitor of APC.

In summary, the studies here show that both PCI and α,AT are physiologic inhibitors of APC in baboons and suggest that when PCI is depleted because of either APC or other proteases, α,AT becomes an even more important inhibitor of APC. Moreover, auxiliary inhibitors, such as α,macroglobulin, also interact with APC in vivo in baboons.

ACKNOWLEDGMENT

The authors thank Anthony Potente and John Harrington for technical assistance and Laurie Thompson for secretarial assistance.

REFERENCES

In vivo and in vitro complexes of activated protein C with two inhibitors in baboons

F Espana, A Gruber, MJ Heeb, SR Hanson, LA Harker and JH Griffin