Impaired Anticoagulant Response to Infusion of Thrombin in Atherosclerotic Monkeys Associated With Acquired Defects in the Protein C System

Steven R. Lentz, José A. Fernández, John H. Griffin, Donald J. Piegors, Rochelle A. Erger, M. René Malinow, Donald D. Heistad

Abstract—To examine the effects of atherosclerosis on the protein C anticoagulant pathway in vivo, we measured anticoagulant responses to intravenous administration of human α-thrombin or activated protein C (APC) in cynomolgus monkeys. Two groups of monkeys were fed either a control diet (n=18) or an atherogenic diet (n=12) that produces both hypercholesterolemia and moderate hyperhomocyst(e)inemia. A third group (n=8) was fed an atherogenic diet for 15 months, and then fed the atherogenic diet supplemented with B vitamins for 6 months to correct the hyperhomocyst(e)inemia. The plasma homocyst(e)ine level was higher in monkeys fed the atherogenic diet (9.6±1.0 μmol/L) than in monkeys fed the control diet (3.7±0.2 μmol/L) or the atherogenic diet with B vitamins (3.6±0.2 μmol/L) (P<0.001). Infusion of thrombin produced a much greater prolongation of the activated partial thromboplastin time in monkeys fed the control diet (52±10 seconds) than in monkeys fed the atherogenic diet either with (24±4 seconds) or without (27±5 seconds) supplemental B vitamins (P<0.02). Thrombin-dependent generation of circulating APC was higher in control (294±17 U/mL) than in atherosclerotic (240±14 U/mL) monkeys (P<0.05), although levels of fibrinogen, plasminogen, D-dimer, and thrombin-antithrombin complexes were similar in each group. Injection of human APC produced a similar prolongation of the activated partial thromboplastin time in control (31±3 seconds) and atherosclerotic (29±2 seconds) monkeys. These findings provide evidence for impaired anticoagulation, due partly to decreased formation of APC, in atherosclerosis. The blunted anticoagulant response to thrombin in hypercholesterolemic monkeys was not corrected by supplementation with B vitamins. (Arterioscler Thromb Vasc Biol. 1999;19:1744-1750.)

Key Words: atherosclerosis ■ cholesterol ■ homocysteine ■ protein C ■ thrombomodulin

Thrombin, a major regulator of hemostasis, has both procoagulant and anticoagulant properties.1 Procoagulant effects of thrombin include proteolytic activation of factors V, VIII, and XI; cleavage of fibrinogen to form a fibrin clot; and stimulation of platelet aggregation. A major anticoagulant effect of thrombin involves activation of protein C, a vitamin K–dependent plasma anticoagulant. Thrombin’s procoagulant and anticoagulant activities are regulated by thrombomodulin, a cofactor that is expressed on the luminal surface of vascular endothelium.2 When bound to thrombomodulin, thrombin’s ability to catalyze procoagulant reactions is inhibited, but its ability to activate protein C is enhanced >1000-fold.3

The protein C anticoagulant pathway is impaired in patients with inherited deficiencies of protein C or protein S and also in patients with a mutation in factor V (factor V Leiden) that renders it resistant to activated protein C (APC).4,5 The physiologic importance of the protein C anticoagulant pathway is underscored by the observation that resistance to APC is found in 20% to 50% of patients with venous thromboembolism.6 Resistance to APC that is independent of factor V Leiden may be associated with increased risk of stroke.7,8

In a previous study, we observed impaired endothelium-dependent vasodilatation and decreased protein C activation in aortas from monkeys with moderate hyperhomocyst(e)inemia [plasma homocyst(e)ine* = 10 μmol/L].9 These observations suggested that endothelial dysfunction, with impaired regulation of thrombin by thrombomodulin, may contribute to an increased risk of atherosclerotic and thrombotic vascular disease in hyperhomocyst(e)inemia.10 The term homocyst(e)ine is used to indicate that plasma homocysteine assays measure the total concentration of thiol, disulfide, and mixed disulfide adducts of homocysteine.

More recently, we examined endothelial function in monkeys fed a diet that produces both hypercholesterolemia and hyperhomocyst(e)inemia.11 In contrast to decreased protein C activation in normocholesterolemic monkeys with isolated...
hyperhomocysteinemia, we observed increased protein C activation in the aorta and carotid artery from atherosclerotic monkeys with the combination of hypercholesterolemia and hyperhomocysteinemia. These ex vivo observations suggested that the regulation of thrombin’s anticoagulant properties may be altered in atherosclerosis, perhaps through increased expression of thrombomodulin in response to vessel injury.

Altered protein C activation may influence the susceptibility of large arteries to chronic or acute thrombotic complications of atherosclerosis, involving small mural thrombi or occlusive thrombus, respectively. The major site of activation of protein C in vivo, however, is thought to be in small vessels, where the local concentration of thrombomodulin is highest. Once generated in the microcirculation, APC is a systemic anticoagulant with a half-life of ~20 minutes. It is not known whether hypercholesterolemia or hyperhomocysteinemia influences thrombin activity or activation of protein C in vivo. The goal of this study, therefore, was to test the hypothesis that anticoagulant responses to -thrombin are altered in atherosclerosis. We used an in vivo approach in which activation of endogenous protein C was measured in response to an infusion of -thrombin in cynomolgus monkeys. Our results indicate that atherosclerosis, with or without moderate hyperhomocysteinemia, significantly alters activation of the protein C pathway in vivo.

Methods

Animals

Adult cynomolgus monkeys (Macaca fascicularis) were fed either a control diet (Purina Monkey Chow,Ralston Purina) or an atherogenic diet that contained 43% of total calories as fat, 0.7% cholesterol, and small amounts of B vitamins, (1 mg vitamin B-6, and 0.3 mg cyanocobalamin, and 20 mg folic acid daily). This atherogenic diet provides both hypercholesterolemia and hyperhomocysteinemia. Another group of monkeys was fed the atherogenic diet for 15 months and then fed the atherogenic diet supplemented with B vitamins (5 mg folic acid, 400 μg cyanocobalamin, and 20 mg pyridoxine hydrochloride daily) for 6 months to decrease plasma homocysteine concentrations. At the end of the feeding period, the weights of the monkeys were 5.9±0.4 kg (control diet), 6.7±0.4 kg (atherogenic diet), and 6.4±0.3 kg (atherogenic diet supplemented with B vitamins).

Experimental Protocol

Human -thrombin (3050 U/mg) was purchased from Enzyme Research Laboratories. Protein C was purified from human plasma by immunoaffinity chromatography as described. Protein C was activated in vitro with human thrombin, and APC was purified by anion exchange chromatography. The purified APC contained no detectable thrombin (<0.05 U of thrombin per mg of APC).

Before administration of thrombin or APC, animals were sedated with ketamine hydrochloride (20 mg/kg IM) and anesthetized with sodium pentobarbital (20 mg/kg IV). A nonobstructive catheter was inserted into an axillary artery for blood sampling, and the axillary vein was cannulated for administration of thrombin, APC, and supplemental anesthesia (sodium pentobarbital 5 mg · kg⁻¹ · h⁻¹). Blood pressure was monitored continuously. Human -thrombin (1.0 to 2.5 μg · kg⁻¹ · min⁻¹) was infused in 10 mL of saline for 10 minutes through the axillary vein catheter. Human APC (50 to 100 μg/kg IV) was injected as a bolus in 5 mL of saline with 5% dextrose. Blood was collected from the axillary artery catheter directly into 3.4 mmol/L EDTA [for measurement of blood counts, homocysteine, and lipids], a 1/10 volume of 3.8% sodium citrate with 3.0 mmol/L benzamidine (for determination of protein C and APC), or a 1/10 volume of 3.8% sodium citrate without benzamidine (for other hemostatic assays). Blood samples were placed immediately on ice, and plasma was isolated by centrifugation at 2500g for 30 minutes at 4°C. Aliquots of plasma were stored at −70°C for up to 20 months before analysis. The protocol was approved by the University of Iowa and Veterans Affairs Animal Care and Use Committees.

Hemostatic Assays

Plasma circulating APC and total protein C were measured by enzyme capture assay using the anti-human protein C light-chain monoclonal antibody C37 and chromogenic substrate S-2366 (Chromogenix). This assay has been used previously to measure APC and protein C in human and baboon plasma. After determination of immunocaptured APC amidolytic activity, immobilized protein C zymogen was activated with 1.0 U/mL Protac (American Diagnostica Inc), and total protein C amidolytic activity was determined as described. Standard curves were generated using plasma pooled from 19 monkeys. One unit of APC or protein C was defined as the amount present in 1.0 mL of pooled monkey plasma. Compared with pooled human plasma, pooled monkey plasma contained 1.7-fold higher total protein C activity. This difference in activity could be due to either a higher concentration of protein C in monkey plasma or a higher amidolytic activity of monkey APC. The concentration of APC was <0.1% of total protein C in pooled monkey plasma.

The activated partial thromboplastin time (APTT) was measured in an ACL-300+ coagulometer (Instrumentation Laboratory) with the Platelin L reagent (Organon Tecknika Corp). Fibrinogen was measured in a fibrometer (BBL Fibrosystem, Becton Dickinson) by the Clauss method with 100 U/mL bovine thrombin (Dade International, Inc). A standard curve was generated using purified human fibrinogen (Chromogenix).

Plasminogen was measured after activation with streptokinase (Kabi Pharmacia, Inc) with the use of the chromogenic substrate S-2251 (Chromogenix). -Antiplasmin was detected by measuring the inhibition of S-2251 hydrolysis by exogenous plasmin (Chromogenix). Plasma activities of factors V, VIII, IX, XI, and XII were measured in an ACL-300+ coagulometer with factor V– or VIII– deficient plasma (George King Bio-Medical). Reference curves for plasminogen, -antiplasmin, and factors V, VIII, IX, XI, and XII were generated using pooled normal monkey plasma that contained, by definition, 1.0 U/mL of each plasma factor. Plasma levels of thrombin-antithrombin complexes and D-dimer were measured by enzyme immunoassay (Behring Diagnostics Inc).

In vitro assays for resistance to APC were performed by adding purified human APC (10.6 μg/mL) to the APTT calcium reagent and measuring the inhibition of S-2251 hydrolysis by exogenous plasmin (Chromogenix). Plasma activities of factors V, VIII, IX, XI, and XII were measured in an ACL-300+ coagulometer with factor V– or VIII– deficient plasma (George King Bio-Medical). Reference curves for plasminogen, -antiplasmin, and factors V, VIII, IX, XI, and XII were generated using pooled normal monkey plasma that contained, by definition, 1.0 U/mL of each plasma factor. Plasma levels of thrombin-antithrombin complexes and D-dimer were measured by enzyme immunoassay using a monoclonal anti-PCI antibody (generously provided by Dr Bonno Bouma, University Hospital, Utrecht, The Netherlands). Peroxidase-labeled polyclonal anti-PCI IgG was used for detection, and the assay was standardized against pooled monkey plasma.

Other Assays

Fasting plasma homocyst(e)ine concentration was measured by high-performance liquid chromatography and electrochemical detection as described previously. Total plasma cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides were measured using methods established by the Lipid Research Centers and standardized by the Centers for Disease Control and Prevention, Atlanta, Ga.

Statistical Analysis

Comparisons between monkeys fed the control or atherogenic diet were performed using the unpaired 2-tailed Student’s t test. Comparisons between multiple groups were performed using 1-way ANOVA. A value of P<0.05 was used to define statistical significance.

Results

Three groups of monkeys were studied. One group (n=18) was fed the control diet. A second group (n=12) was fed the atherogenic diet for 21.1±1.2 months (mean±SE). The third
group (n=8) was fed the atherogenic diet for 14.5±0.8 months and then fed the atherogenic diet supplemented with B vitamins for 5.6±0.4 months (total time on the atherogenic diet was 20.0±1.0 months). Plasma homocyst(e)ine was elevated (9.6±1.0 μmol/L) in monkeys fed the atherogenic diet without B vitamin supplementation (P<0.001 versus control diet) but not in monkeys fed atherogenic diet with B vitamins (Figure 1A). Plasma total cholesterol was elevated (>400 mg/dL) in monkeys fed atherogenic diet with or without B vitamin supplementation (P<0.001 versus control diet; Figure 1B). Elevation of total cholesterol in these animals was caused by the elevation of LDL cholesterol; plasma levels of HDL cholesterol were lower in monkeys fed atherogenic diets than in monkeys fed the control diet, and levels of triglycerides were similar in all groups (Table 1).

Response to Infusion of Thrombin
In a pilot experiment, a 10-minute infusion of human α-thrombin produced dose-dependent increases in APTT and circulating APC in a monkey fed the control diet (Figure 2). A dose of thrombin of 2.5 μg · kg⁻¹ · min⁻¹ was chosen to compare anticoagulant responses in monkeys fed control, atherogenic, and vitamin-supplemented atherogenic diets. Baseline APTT values before infusion of thrombin did not differ among the 3 groups (Figure 3A). Infusion of thrombin produced greater prolongation of the APTT in monkeys fed the control diet (prolongation of 52±10 seconds over baseline APTT) than in monkeys fed the atherogenic diet with (24±4 seconds) or without (27±5 seconds) supplemental B vitamins (P<0.02; Figure 3A).

After infusion of thrombin, peak levels of circulating APC were 18% lower in monkeys fed the atherogenic diet without

| TABLE 1. Plasma Lipid Profiles of Monkeys Fed the Control, Atherogenic, or Vitamin-Supplemented Atherogenic Diet |
|-------------------------------------------------|-----------------|-----------------|-----------------|
|                                                  | Atherogenic Diet |                  |                  |
|                                                  | Control Diet (n=8) | Without Vitamins (n=12) | With Vitamins (n=8) |
| LDL cholesterol, mg/dL                          | 40±5             | 466±51*          | 377±25*         |
| HDL cholesterol, mg/dL                          | 64±4             | 30±4*            | 25±5*           |
| Triglycerides, mg/dL                            | 26±5             | 28±10            | 17±10           |

Values are mean±SE. *P<0.05 vs control diet.
supplemental B vitamins (240 ± 14 U/mL) than in monkeys fed the control diet (294 ± 17 U/mL, P = 0.03; Figure 3B). Levels of circulating APC did not differ between monkeys fed the atherogenic diet with supplemental B vitamins and those fed the atherogenic diet without supplemental B vitamins (Figure 3B). These findings suggest that anticoagulant responses to thrombin were impaired in atherosclerotic monkeys in part because of decreased circulating APC.

After infusion of thrombin, platelet count decreased <15%, plasma fibrinogen decreased ~35%, factor V activity decreased 40% to 50%, and factor VIII activity decreased 60% to 70% in each group of monkeys (Table 2). Plasma activities of factors IX, XI, and XII decreased <30%, and total plasma protein C activity decreased <15% after infusion of thrombin in each group (Table 2). No significant differences in the generation of thrombin-antithrombin complexes or D-dimer or in the consumption of fibrinogen, plasminogen, or α2-antiplasmin were observed in the 3 groups (Table 2).

Response to APC In Vitro
One potential mechanism for impaired anticoagulant response to thrombin is resistance to the anticoagulant effects of APC. To determine whether resistance to APC could be detected in plasma from atherosclerotic monkeys in vitro, the APTT of monkey plasma was measured in the absence or presence of 0.6 µg/mL of human APC in an APC resistance assay. Addition of APC prolonged the APTT by 44.2 ± 3.6 seconds in monkeys fed the control diet and by 36.0 ± 2.7 seconds in monkeys fed the atherogenic diet (P > 0.05; Figure 4).

Response to Injection of APC
To determine whether monkeys fed the atherogenic diet exhibited resistance to APC in vivo, we measured anticoagulant responses to injection of purified human APC. Injection of APC into a control monkey produced prolongation of the APTT and elevation of plasma APC that were dose dependent (Figure 5). A bolus (75 µg/kg) of APC produced a similar prolongation of the APTT in monkeys fed control (31 ± 3 seconds) and atherogenic (29 ± 2 seconds) diets (P > 0.05; Figure 6A). Recovery and elimination of injected human APC were similar in the 2 groups, with peak levels of 407 ± 21 and 430 ± 13 U/mL and plasma half-lives of 23.9 ± 1.3 and 24.3 ± 1.3 minutes, respectively (Figure 6B). Thus, the half-life of human APC was not altered by the atherogenic diet.

Because plasma PCI inhibits both activation of protein C and APC activity,21,25 we also measured levels of PCI in monkey plasma. Levels of PCI were similar in monkeys fed the control diet (0.96 ± 0.07 U/mL), the atherogenic diet (1.00 ± 0.08 U/mL), and the atherogenic diet supplemented with B vitamins (0.94 ± 0.08 U/mL).

Discussion
To examine the effects of diet-induced hypercholesterolemia and hyperhomocysteinemia on the protein C anticoagulant pathway in vivo, we infused human α-thrombin in low doses into control and atherosclerotic cynomolgus monkeys. Our major findings are that anticoagulant responses to thrombin are impaired in monkeys with diet-induced atherosclerosis and that supplementation with B vitamins does not restore anticoagulant responses to normal in atherosclerotic monkeys with persistent hypercholesterolemia. Decreased responses to thrombin in atherosclerotic monkeys were associated with decreased generation of APC in vivo. These findings suggest

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**TABLE 2. Hemostatic Parameters at Baseline and 90 Minutes After Infusion of Thrombin Into Monkeys Fed the Control, Atherogenic, or Vitamin-Supplemented Atherogenic Diet**

<table>
<thead>
<tr>
<th></th>
<th>Control Diet</th>
<th>Atherogenic Diet</th>
<th>Atherogenic Diet With Vitamins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n Baseline</td>
<td>90 Minutes</td>
<td>n Baseline</td>
</tr>
<tr>
<td>Platelet count, ×10³/µL</td>
<td>18 350 ± 18</td>
<td>339 ± 19</td>
<td>12 392 ± 38</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>18 193 ± 7</td>
<td>123 ± 10</td>
<td>12 148 ± 9</td>
</tr>
<tr>
<td>Factor V, U/mL</td>
<td>16 0.89 ± 0.10</td>
<td>0.51 ± 0.06</td>
<td>12 1.17 ± 0.10</td>
</tr>
<tr>
<td>Factor VIII, U/mL</td>
<td>16 1.00 ± 0.08</td>
<td>0.39 ± 0.07</td>
<td>12 0.94 ± 0.07</td>
</tr>
<tr>
<td>Factor IX, U/mL</td>
<td>15 1.02 ± 0.09</td>
<td>0.99 ± 0.14</td>
<td>12 0.98 ± 0.09</td>
</tr>
<tr>
<td>Factor XI, U/mL</td>
<td>18 0.78 ± 0.06</td>
<td>0.62 ± 0.05</td>
<td>12 0.62 ± 0.05</td>
</tr>
<tr>
<td>Factor XII, U/mL</td>
<td>16 0.86 ± 0.05</td>
<td>0.70 ± 0.05</td>
<td>11 0.86 ± 0.08</td>
</tr>
<tr>
<td>Protein C, U/mL</td>
<td>18 1.10 ± 0.07</td>
<td>1.05 ± 0.06</td>
<td>12 1.24 ± 0.06</td>
</tr>
<tr>
<td>Thrombin-antithrombin complex, ng/mL</td>
<td>17 11 ± 5</td>
<td>83 ± 30</td>
<td>11 14 ± 3</td>
</tr>
<tr>
<td>D-dimer, µg/mL</td>
<td>18 0.94 ± 0.49</td>
<td>112 ± 55</td>
<td>12 0.82 ± 0.30</td>
</tr>
<tr>
<td>Plasminogen, U/mL</td>
<td>8 0.73 ± 0.05</td>
<td>0.73 ± 0.05</td>
<td>12 0.85 ± 0.09</td>
</tr>
<tr>
<td>α2-Antiplasmin, U/mL</td>
<td>8 1.02 ± 0.02</td>
<td>0.95 ± 0.04</td>
<td>12 0.96 ± 0.04</td>
</tr>
</tbody>
</table>

Values are mean ± SE. *P < 0.05 vs control diet.
that impairment of the protein C anticoagulant pathway may contribute to thrombogenicity in atherosclerosis.

After its intravenous infusion, thrombin is rapidly cleared from the circulation, and some of it binds to thrombomodulin and activates protein C.2,3,18,26 Although thrombomodulin is expressed on the endothelial surface of both large and small vessels, most protein C is activated in vivo in small vessels, where the ratio of endothelial surface area to plasma volume is highest.2 In baboons, infusion of thrombin causes extensive activation of protein C and is associated with a systemic antithrombotic response that can be demonstrated by a decreased accumulation of fibrin and platelets on a thrombogenic vascular graft.15,16,18 This antithrombotic response can be completely abated by pretreatment with a monoclonal antibody that blocks thrombin-dependent protein C activation.19 Infusion of thrombin also prolongs the APTT and generates anticoagulant APC in cynomolgus monkeys.27

The monkeys in this study were fed an atherogenic diet for \(~\approx 20\) months. In previous studies, we demonstrated that this diet produces both hyperhomocyst(e)inemia and hypercholesterolemia, with moderately severe atherosclerotic lesions in large vessels.11,28 We anticipated that infusion of thrombin might generate higher levels of APC in atherosclerotic monkeys than in monkeys fed the control diet, because we previously observed increased protein C activation in explants of aorta and carotid artery from atherosclerotic monkeys.11 In contrast to our expectations, however, levels of circulating APC were almost 20% lower after infusion of thrombin in atherosclerotic monkeys than in control monkeys. These observations suggest that although local activation of protein C may be increased in large vessels that contain atherosclerotic lesions, systemic generation of APC, which occurs predominantly in small vessels, may be decreased in atherosclerotic animals.

Atherosclerotic monkeys did not exhibit significant resistance to human APC in vitro (Figure 4), and injection of human APC produced a similar prolongation of the APTT in monkeys fed control and atherogenic diets (Figure 6). The plasma half-life of human APC was \(~\approx 24\) minutes in both control and atherosclerotic monkeys. This calculated half-life is similar to that estimated for endogenous APC in baboons (\(~\approx 16\) minutes) in our previous studies18 and in cynomolgus monkeys (\(~\approx 15\) minutes) in a previous report.27 Thus, differences in clearance or inactivation of APC do not account for differences in anticoagulant response to thrombin between control and atherosclerotic monkeys.

After infusion of thrombin into monkeys, there was a significantly decreased activation of protein C associated with atherosclerosis and hypercholesterolemia, since peak levels of circulating APC were \(~\approx 20\%) lower in atherosclerotic monkeys compared with control monkeys (Figure 3B). To help determine whether this modest difference in plasma APC could account for the large difference in APTT prolongation observed in response to thrombin (Figure 3A), we examined the relationship between circulating APC levels and APTT by using data obtained after administration of either APC or thrombin (Figure 7). After injection of APC, the APTT was similarly and directly related to the level of circulating APC in monkeys fed either the control or the atherogenic diets (Figure 7A). During the first 5 to 10 minutes after beginning the infusion of thrombin, the relationship between APTT and circulating APC was very similar to that observed after injection of APC (Figure 7B). At later times after thrombin infusion, however, there was a leftward shift in the APTT versus APC dose-response relationship, with a much greater prolongation of the APTT than can be accounted for by plasma APC alone (Figure 7B). Furthermore, this shift in response to APC was significantly blunted in atherosclerotic monkeys compared with control monkeys (Figure 7B). These findings suggest that the impaired anticoagulant response to thrombin in atherosclerotic monkeys is caused partly by diminished generation of APC and partly by
whereas thrombomodulin is expressed on both large and small blood vessels. 

29 EPCR is present mainly on large blood vessels, whereas thrombomodulin-dependent activation of protein C by thrombin, thrombomodulin, and the Control of Hemostasis. Austin, Tex: RR Landes Co; 1994;1:91–120. 

337–338.


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doi: 10.1161/01.ATV.19.7.1744

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