Anticoagulant Responses to Thrombin Are Enhanced During Regression of Atherosclerosis in Monkeys

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Background—Diet-induced atherosclerosis in monkeys produces abnormal anticoagulant responses to thrombin, including decreased generation of activated protein C (APC). We tested the hypothesis that anticoagulant responses to thrombin increase toward normal during regression of atherosclerosis.

Methods and Results—Six cynomolgus monkeys were fed a high-fat atherogenic diet for 44 months and then a low-fat regression diet for 8 months. Serum total cholesterol decreased from 417±44 to 68±6 mg/dL (mean±SEM) and LDL cholesterol decreased from 375±44 to 27±5 mg/dL after the regression diet. In response to infusion of thrombin, the activated partial thromboplastin time (APTT) increased by 11±3 seconds before the regression diet and by 41±22 seconds after the regression diet (P=0.01). The peak level of circulating plasma APC was 52±9 ng/mL before the regression diet and 88±17 ng/mL after the regression diet (P=0.01). The APC sensitivity of plasma factor V was identical before and after the regression diet. Three additional atherosclerotic monkeys that remained on the high-fat diet for 8 months demonstrated no change in APTT or activation of protein C in response to thrombin.

Conclusions—Short-term dietary regression of atherosclerosis produces enhanced anticoagulant responses to thrombin in vivo. (Circulation. 2002;106:842-846.)

Key Words: atherosclerosis • endothelium • coagulation • thrombin

Reduction of cholesterol levels in patients with established coronary artery disease produces regression of atherosclerotic lesions and markedly decreases the risk of ischemic coronary events.1–3 The clinical benefit of cholesterol lowering occurs before structural regression of atherosclerosis.1 When atherosclerotic monkeys are fed a regression diet, improvement in endothelial function (demonstrated by enhanced relaxation to endothelium-dependent vasodilators) precedes improvement in vascular structure.4–5 Endothelial vasomotor function also improves rapidly (within a few months) during cholesterol-lowering therapy in humans.6 It is not known, however, whether regression of atherosclerosis produces beneficial effects on anticoagulant pathways, particularly those involving interactions of coagulation factors with components of the vascular wall. If so, such effects could contribute to the clinical benefit of cholesterol-lowering therapy.

Thrombin is a major regulator of procoagulant and anticoagulant pathways.7 Procoagulant effects of thrombin include cleavage of fibrinogen to generate fibrin, activation of platelets via protease-activated receptors, and feedback activation of coagulation factors V, VIII, and XI. The major anticoagulant effect of thrombin is activation of protein C, which is dependent on the endothelial cofactors thrombomodulin and endothelial protein C receptor (EPCR).8,9 Recent studies indicate that the activity of the protein C anticoagulant pathway may be impaired in atherosclerosis.10 For example, the combination of decreased HDL cholesterol and increased LDL cholesterol, which is common in patients with atherosclerosis, may negatively influence the anticoagulant activity of activated protein C (APC).11 In a previous study, we found that activation of protein C in response to infusion of thrombin was blunted in monkeys with diet-induced atherosclerosis.12 The objective of the present study was to test the hypothesis that the protein C anticoagulant response to thrombin increases toward normal during short-term dietary regression of atherosclerosis in nonhuman primates.

Methods

Experimental Protocol

Nine adult cynomolgus monkeys (Macaca fascicularis) (Biomedical Research Foundation, Houston, Tex) were fed an atherogenic diet that contained 0.7% cholesterol and 43% of total calories as fat.13,14 These animals were part of a larger group of monkeys that were examined for effects of regression of atherosclerosis on vascular superoxide, as reported elsewhere.3 After 44±1 months on the atherogenic diet, the 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 and supplemental anesthesia (sodium pentobarbital 5 mg/kg per hour). Human α-thrombin (Enzyme Research Laboratories) (25 μg/kg) was infused in 10 mL of saline over 10 minutes through the axillary vein catheter, as described previously.12,15 Blood was collected at intervals (0 to 90 minutes after beginning infusion of thrombin) from the axillary artery catheter directly into a 1/10 volume of 3.8% sodium citrate with 0.3 mol/L benzamidine (for determination of APC) or 3.8% sodium citrate without benzamidine (for other hemostatic assays). Blood samples were placed immediately on ice, and plasma was isolated by centrifugation at 2500 g for 30 minutes at 4°C. Additional blood samples were collected into serum separator tubes for determination of cholesterol or 3.4 mmol/L EDTA for determination of total plasma homocysteine (tHcy).

Six of the monkeys were then fed a low-fat regression diet,2 whereas 3 monkeys remained on the atherogenic diet. After 8 months, the animals were again sedated and anesthetized, and the infusion of thrombin was repeated. After a recovery period of at least 1 week, the animals were euthanized by administration of sodium pentobarbital (200 mg/kg IV). Lung tissue was harvested for protein C activation assays, and arterial tissue was harvested for morphometric and vasomotor studies.3 Lung tissue from 7 additional atherosclerotic monkeys5 was used for protein C activation assays. The protocol was approved by the University of Iowa and Veterans Affairs Animal Care and Use Committees.

### Hemostasis Assays

Plasma APC was measured by enzyme capture assay using the anti-human protein C light-chain monoclonal antibody C316 and chromogenic substrate S-2366, as described previously.12 The assay was standardized using a pool of normal monkey plasma that contained ~6.5 ng/mL APC. Compared with pooled human plasma, pooled monkey plasma contained 3-fold higher APC activity. The activated partial thromboplastin time (APTT) was measured in an ACL-300+ coagulometer (Instrumentation Laboratory) using the Platelet L reagent (Organon Tecknika Corp). For some analyses, the C3 monoclonal antibody was added (final concentration, 40 μg/mL) before measuring the APTT. This concentration of the C3 antibody inhibited the activity of APC in Protac-activated pooled monkey plasma by 90% (data not shown). Plasma levels of thrombin-antithrombin complexes were measured by enzyme immunoassay (Behring Diagnostics Inc). To measure the APC sensitivity of plasma factor V, monkey plasma was diluted 1:10 into human factor V–deficient plasma (George King Bio-Medical, Inc). The APTT was then performed in the presence of 0 to 20 nmol/L human APC (Enzyme Research Laboratories), as described previously.12

### Protein C Activation

The ability of lung lysates to enhance the activation of protein C (thrombomodulin activity) was measured using a 2-stage assay described previously.17,18 Lung tissue was homogenized in 0.02 mol/L Tris-HCl and 0.1 mol/L NaCl (pH 8.0) and incubated in 1.0% Triton X-100 for 10 minutes at room temperature. Lysates were then incubated for 60 minutes with 2.6 nmol/L human thrombin (Enzyme Research Laboratories) and 0.15 μmol/L rat thrombomodulin (Kabi Pharmacia Hepar, Inc). The reaction was stopped by addition of heparin and antithrombin, and the amidolytic activity of APC was measured spectrophotometrically. Reference curves were generated using rabbit lung thrombomodulin (American Diagnostica). One unit of activity was defined as the amount of APC generated in the presence of 1.0 nmol/L rabbit thrombomodulin (Kabi Pharmacia Hepar, Inc). The protein content of the lysates was measured using a modified Bradford method (Bio-Rad Laboratories).

### Other Assays

Serum total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides were measured in the clinical laboratories of the University of Iowa Hospitals. Plasma tHcy, defined as the total concentration of homocysteine after quantitative reductive cleavage of all disulfide bonds,19 was measured in the University of Iowa General Clinical Research Center by high-performance liquid chromatography.20

### Statistical Analysis

The paired, 2-tailed Student’s t test was used to compare values obtained before and after 8 months of the regression diet. Unpaired Student’s t tests were used to compare values for thrombomodulin activity between control, atherosclerotic, and regression groups. APTT and APC responses to thrombin were analyzed using 2-way repeated-measures ANOVA with Bonferroni multiple-comparison analysis. A value of P<0.05 was used to define statistical significance. Values are reported as mean±SEM.

## Results

### Body Weight, Cholesterol, and tHcy

After 8 months on the regression diet, the weights of the monkeys did not change significantly (Table). Serum total cholesterol decreased from 417±44 to 68±6 mg/dL (P<0.001), LDL cholesterol decreased from 375±44 to 27±5 mg/dL (P<0.001), and plasma tHcy decreased from 7.3±0.8 to 4.5±0.5 μmol/L (P<0.01). HDL cholesterol and triglycerides did not change significantly.

### Morphometry and Vasomotor Responses

As reported elsewhere,5 the atherosclerotic monkeys had advanced neointimal lesions of the femoral artery before the regression diet. No significant decreases in intimal area or intima to media ratio were detected after 8 months of the regression diet.3 Despite the persistence of atherosclerotic lesions, relaxation of the femoral artery to acetylcholine increased toward normal after the regression diet. For the 6 animals reported here, maximal relaxation of the femoral artery to acetylcholine was 74±5% before the regression diet and 95±6% after the regression diet (P=0.05).

### Infusion of Thrombin

Infusion of thrombin in monkeys produces activation of protein C and prolongation of the APTT,21 and these responses are impaired in atherosclerotic monkeys.12,15 To determine whether anticoagulant responses to thrombin increase during regression of atherosclerosis, we measured the APTT and circulating levels of APC in response to infusion of thrombin. Baseline values for APTT and APC before infusion of thrombin did not differ significantly before and after the regression diet. After infusion of thrombin, the APTT increased by 11±3 seconds before the regression diet

<table>
<thead>
<tr>
<th>Effect of Regression Diet on Body Weight, Fasting Lipid Profile, and Total Homocysteine</th>
<th>Before Regression (n=6)</th>
<th>After Regression (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>8.2±0.4</td>
<td>8.5±0.5</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>417±44</td>
<td>68±6*</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>375±44</td>
<td>27±5*</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>37±5</td>
<td>33±2</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>25±7</td>
<td>37±5</td>
</tr>
<tr>
<td>Total homocysteine, μmol/L</td>
<td>7.3±0.8</td>
<td>4.5±0.5*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.05 vs before regression.
and by 41±22 seconds after the regression diet (P=0.01) (Figure 1A). The peak level of circulating plasma APC was 52±9 ng/mL before the regression diet and 88±17 ng/mL after the regression diet (P=0.01) (Figure 1B). Peak plasma levels of thrombin-antithrombin complexes were similar before and after the regression diet (130±5 versus 167±27 ng/mL; P>0.05).

Role of APC

During the first 10 minutes after beginning the thrombin infusion, the APTT was directly related to the plasma APC concentration both before and after the regression diet (Figure 2). At later time points (>20 minutes after beginning the thrombin infusion), there was a leftward shift in the APTT versus APC concentration response relationship, with a greater prolongation of the APTT for a given concentration of APC. This leftward shift in the relationship between APTT and APC was more pronounced after the regression diet, which suggests that regression diet influenced both the amount of APC produced and the degree of prolongation of the APTT for a given concentration of APC.

To address more directly the contribution of APC to the increased APTT response to thrombin, plasma samples were treated with a monoclonal antibody (C3) that blocks the activity of monkey APC. The C3 antibody did not significantly blunt the small increase in the APTT before the regression diet (Figure 3A). After the regression diet, however, the C3 antibody reversed the thrombin-dependent prolongation of the APTT by 60% to 80% (P<0.05 versus control IgG at the 10-minute time point) (Figure 3B), which provides support for the conclusion that activation of endogenous APC is a major mechanism for prolongation of the APTT after infusion of thrombin.

To determine whether the anticoagulant activity of APC was influenced by the regression diet, the APC sensitivity of plasma factor V was measured after 1:10 dilution in human factor V–deficient plasma. The sensitivity of factor V to APC

Figure 1. Anticoagulant response to infusion of thrombin. APTT (A) and circulating APC (B) were measured before, during, and after a 10-minute infusion of human thrombin before the regression diet (●; n=6) and after 8 months of regression diet (○; n=6). Values are mean±SEM. *P<0.05 vs before regression diet.

Figure 2. Relationship between APTT and circulating APC after infusion of thrombin. Values for APTT and APC were taken from the data shown in Figure 1 and represent the mean values of samples collected at various times after infusion of thrombin. ●, before the regression diet; ○, after the regression diet. The arrows depict the path of the time course.

Figure 3. Effect of APC blocking antibody on APTT response to infusion of thrombin. The APTT of plasma samples from the animals shown in Figure 1, collected at the indicated times after infusion of thrombin, was measured in the presence of control murine IgG (open bars) or the C3 murine monoclonal antibody that blocks APC activity (filled bars). A, Before regression diet (n=4). B, After 8 months of regression diet (n=6). Values are mean±SEM. *P<0.05 vs control IgG.

Figure 4. APC sensitivity of plasma factor V. Monkey plasma collected before the regression diet (●; n=6) or after 8 months of regression diet (○; n=6) was diluted 1:10 in human factor V–deficient plasma, and the APTT was measured in the presence of the indicated concentrations of human APC.
was identical before and after the regression diet (Figure 4), which suggests that the major effect of the regression diet on the protein C pathway was increased generation of APC, rather than increased sensitivity to APC.

**Time Controls**

Three atherosclerotic monkeys were maintained on the atherogenic diet for an additional 8 months. In contrast to the monkeys fed the regression diet, these animals demonstrated no change in relaxation of the femoral artery to acetylcholine. They also had no change in anticoagulant responses to thrombin; after infusion of thrombin, the APTT increased by 10±2 seconds at the time of the initial study and by 10±6 seconds after the additional 8 months of the regression diet ($P>0.05$).

**Thrombomodulin Activity in Lung**

Thrombomodulin activity was determined from the ability of lung lysates to enhance the activation of protein C by thrombin\(^{17,18}\) and was measured for a group of atherosclerotic monkeys fed the atherogenic diet for 48±5 months (n=7) and for the group of atherosclerotic monkeys after 8 months of regression diet (n=6). The thrombomodulin activity of the regression group (1.29±0.22 U/mg) did not differ significantly from that of the atherosclerotic group (1.13±0.18 U/mg) ($P>0.05$).

**Discussion**

The major new finding of this study is that anticoagulant responses to thrombin are enhanced during dietary regression of atherosclerosis in monkeys. Previous studies of regression of atherosclerosis have demonstrated that vasomotor responses return toward normal within a few months, well before detectable morphometric evidence of reduction in atherosclerotic lesions\(^4\)\(^\text{–}\)\(^6\). Here we have found that anticoagulant responses to thrombin, which are impaired in atherosclerotic monkeys,\(^{12,13}\) also increase relatively quickly (within 8 months) when monkeys are fed a low-fat regression diet.

Thrombotic mechanisms are thought to play a major role in the pathophysiology of acute coronary syndromes and other complications of atherosclerosis.\(^{22}\) A variety of epidemiological studies demonstrate an association between hypercholesterolemia and systemic markers of hypercoagulability.\(^{10,23}\) Although the molecular mechanisms responsible for this association are incompletely understood, there is evidence that the protein C anticoagulant pathway is altered in hypercholesterolemia. The anticoagulant activity of APC is enhanced by HDL cholesterol but not LDL cholesterol,\(^{11}\) which suggests a possible mechanism for protection from thrombotic events in patients with elevated HDL cholesterol. APC activity also may be enhanced by other components of plasma lipoproteins, including cardioliopin,\(^{24}\) glucosylceramide,\(^{25}\) and certain oxidized phospholipids.\(^{26}\) Other lipoproteins, including triglyceride-rich lipoproteins and oxidized LDL, may accelerate procoagulant reactions.\(^{10,22}\) These observations indicate that alterations in plasma lipoproteins during atherosclerosis may influence both anticoagulant and procoagulant pathways.

We found that the APC sensitivity of plasma factor V was identical before and after the regression diet, a finding that is in accordance with previous results in which atherosclerosis did not produce resistance to infused human APC.\(^{12}\) We did, however, observe increased circulating APC activity as well as an increase in the APTT versus APC concentration-response relationship after infusion of thrombin in monkeys fed the regression diet (Figure 2). Most, but not all, of the thrombin-induced prolongation of the APTT could be prevented by an anti-APC–blocking antibody (Figure 3). These results strongly suggest that the major effect of the regression diet on the anticoagulant response to thrombin is mediated by increased generation of APC. The residual prolongation of the APTT that was not suppressed by the blocking antibody may reflect a minor component of the APTT response to thrombin that does not require APC activity.\(^{12}\)

Activation of protein C is dependent on the endothelial cofactors thrombomodulin and EPCR.\(^{8,9}\) After its intravenous infusion, thrombin is rapidly cleared from the circulation, partly through its high-affinity binding interaction with thrombomodulin in the pulmonary vasculature.\(^{28}\) It is unlikely that the different anticoagulant responses to thrombin before and after the regression diet were caused by differences in recovery or clearance of thrombin, because similar levels of thrombin-antithrombin complexes were observed in both groups.

Thrombomodulin is susceptible to inactivation by reactive oxygen species such as superoxide,\(^{29}\) so one possible mechanism for increased generation of APC during regression of atherosclerosis is increased pulmonary thrombomodulin activity attributable to decreased levels of vascular superoxide.\(^{5}\) Another possible mechanism for enhanced generation of APC during regression of atherosclerosis is increased expression or availability of EPCR. EPCR seems to have a major influence on anticoagulant responses to thrombin in vivo, because activation of protein C after infusion of thrombin in baboons was inhibited >80% by a monoclonal anti-ECPR antibody.\(^{30}\) The EPCR promoter contains a thrombin response element,\(^{31}\) which raises the possibility that downregulation of thrombin receptor signaling may lead to decreased expression of EPCR in atherosclerosis. We did not, however, observe a significant difference in protein C activation when lung lysates containing thrombomodulin from atherosclerotic and regression monkeys were analyzed in vitro. It remains possible that the activity of thrombomodulin in other, nonpulmonary vascular beds may have been affected by the regression diet and that these effects could have contributed to increased activation of protein C. An additional possibility is that atherogenic lipoproteins or other components of hyperlipidemic plasma may alter the activation of protein C or APC activity in vivo and that these effects were not apparent when thrombomodulin activity was measured in lung lysates in vitro. Future studies will be needed to address these potential mechanisms.

We also considered the possibility that enhanced anticoagulant responses to thrombin after the regression diet could have been related to a decrease in plasma tHcy concentration. Hyperhomocysteinemia can produce impairment of endothelial function, including thrombomodulin-dependent activation of protein C.\(^{32}\) In the present study, we found that plasma
Hcy levels decreased significantly after the regression diet. This finding is concordant with previous studies in which we have found that the atherogenic diet produces mild hyperhomocystinemia in monkeys.\textsuperscript{12,13,33} However, correction of hyperhomocystinemia does not restore normal anticoagulant responses to thrombin in atherosclerotic monkeys,\textsuperscript{12,13} so it is very unlikely that the small decrease in plasma Hcy had any influence on the response to thrombin after the regression diet.

In summary, we have found that anticoagulant responses to thrombin increase during diet-induced regression of atherosclerosis in monkeys. This effect was mediated in large part by increased thrombin-dependent generation of APC. Like improvement in endothelium-dependent vasoromotor responses,\textsuperscript{4,5} enhancement of anticoagulant responses occurred relatively rapidly (within 8 months) when atherosclerotic monkeys were fed a regression diet. Normalization of impaired endothelial function during regression of atherosclerosis has prognostic implications in predicting adverse clinical outcomes.\textsuperscript{34,35} Our results suggest that enhanced activity of endogenous anticoagulant pathways also may contribute to the clinical benefit of cholesterol-lowering therapy.

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