Kininogens Are Antithrombotic Proteins In Vivo

Robert W. Colman, John V. White, Sherry Scovell, Antoni Stadnicki, R. Balfour Sartor

Abstract—Kininogens have recently been shown to possess antiadhesive, anticoagulant, and profibrinolytic properties and can inhibit platelet activation at low thrombin concentrations. To test whether kininogens have antithrombotic properties in vivo, we devised a model of limited arterial injury confined to removal of the endothelium. Brown-Norway Katholiek strain rats with an absence of low- and high-molecular-weight kininogen due to a single point mutation, A163T, were compared in the thrombosis model to the wild-type animals, which were otherwise genetically identical. Despite an equivalent vascular injury, the mean time (±SEM) for a 90% decrease in flow measured by laser Doppler was 38.4±17 minutes in the kininogen-deficient rats compared with 194±29 minutes in the wild-type animals (P<0.002). The degree of vascular injury was the same. No evidence for disseminated intravascular coagulation (decrease in factor V, antithrombin, or fibrinogen) or excessive fibrinolysis (elevation of fibrinogen degradation products) was found in either group of animals. The results suggest that kininogens have antithrombotic properties at low concentrations of thrombin and that inhibitory peptides derived from kininogen may constitute a new antithrombotic strategy. (Arterioscler Thromb Vasc Biol. 1999;19:2245-2250.)

Key Words: arterial thrombosis ■ kininogens ■ antithrombotic ■ fibrinolysis ■ rats

Kininogens are plasma proteins first recognized as the precursors of a peptide, bradykinin, which was liberated by plasma kallikrein.1 Bradykinin can reproduce many inflammatory changes, including edema, pain, vasodilation, and increased vascular permeability. A deficiency of plasma high- (HK) and low- (LK) molecular-weight kininogens2 in humans does not lead to a hemorrhagic disorder, despite a marked prolongation of the activated partial thromboplastin time. In fact, cloning3 and delineation of the structure-function relationships of kininogen have revealed new properties of this protein, including cysteine protease inhibition,4 modulation of thrombin-induced platelet aggregation,5 antiadhesive properties,6 and profibrinolytic potential.7 HK and LK have been divided into domains D1, D2, and D3, which compose the common heavy-chain N-terminal to domain D4, which contains the bradykinin sequence. LK contains a small domain, D5L , whereas HK contains the large domains D5 H and D6, which compose the light chains.3 The ability of D2 of the kininogens to inhibit platelet calpain8 has been linked to an inhibition of thrombin-induced platelet aggregation,9 whereas inhibition of the binding of thrombin to platelets by D3 of kininogens has led to a 10-fold shift in the dose-response curve of thrombin-induced platelet activation.9 HK can compete for fibrinogen binding to neutrophils10 by competing for a site on the integrin Mac-1 and thus, limit the adhesion of neutrophils to artificial and biological surfaces. Peptides from D6 of HK can downregulate urokinase-dependent plasminogen activation on human endothelial cells7 by inhibiting the binding of prekallikrein to HK.

All of these studies suggest that HK should serve as an antithrombotic protein. Conversely, individuals deficient in HK should manifest a prothrombotic state. Unfortunately, HK deficiency is a rare condition, and only a very small number of individuals have been observed in a serial fashion. Therefore, to test this hypothesis, we decided to use a well-defined animal model. Fortunately, a natural “knockout” exists in the Katholiek strain of the Brown-Norway rat. These rats have absent plasma HK and LK due to a single point mutation in the heavy chain, Ala-163 to Thr, which results in the defective hepatic secretion of both HK and LK.12 This mutation is the only defect, and the wild-type Brown-Norway rat has an identical genetic background except for this single amino acid change in kininogens.

We developed a model of intimal injury limited to intimal loss in an artery sufficiently large to permit blood flow measurements. We compared the response to injury in the Katholiek strain and the wild-type Brown-Norway rat to test the hypothesis that HK functions as an antithrombotic protein.

Methods

Model of Arterial Thrombosis

Ten Brown-Norway rats, 5 wild-type and 5 kininogen deficient of both sexes (250 to 350 g in weight; Charles River Laboratories,
Wilmington, Mass), underwent experimental surgery. Animal care complied with the Principles of Laboratory Animal Care (formulated by the National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985) and was approved by the Temple University Committee on Animal Research. The rats were initially anesthetized with isofluorane, underwent tracheostomy, and were placed on 2.5% isofluorane throughout the experiments. ECG monitoring was continuous throughout. The left common carotid artery was exposed at the base of the neck and cannulated in retrograde fashion with a 22-gauge plastic cannula for continuous monitoring of systemic pressures by using a small-animal/small-volume transducer. The right femoral artery was also exposed and cannulated in a retrograde manner with a 22-gauge plastic cannula for continuous pressure monitoring distal to the injured segment of the aorta. The abdomen was then entered through a midline incision, and the bowel was reflected to the right to expose the aorta. The periaortic tissues were dissected free from the aorta from the renal arteries to the iliac arteries bilaterally. Baseline aortic blood flow was measured by laser Doppler and recorded. A mild vascular injury was used, in which the endothelium was removed without significant structural damage to the media. Specifically, a disposable pulmonary cytology brush (Mill-Rose Laboratories, Inc) with a 1-mm diameter was inserted through an arteriotomy in the left iliac artery, and the entire infrarenal aorta was brushed and denuded of its endothelium. Immediately after the injury, the brush was removed, the left iliac artery was ligated, and aortic blood flow was measured and recorded at 5-minute intervals just proximal to the aortic bifurcation. The point of termination of the measurements was defined as a reduction in baseline blood flow >90% or a total time limit of 240 minutes of monitoring, at which time the animal was killed. If no occlusion was found, the time was recorded as 240 minutes for statistical purposes. On completion of the recordings, an intracardiac puncture was performed, and blood was collected in 1/10 volume of 3.8% sodium citrate (9:1, vol/vol). The samples were centrifuged at 4°C for 15 minutes at 3000 \( \times g \) before the assays were performed.

### Histological Studies

The histology of the midportion of the injured segment of the aortic specimens was reviewed by light microscopy for overall architecture and severity of injury by an observer blinded to the strain of the rat evaluated. Injury severity to the intima, media, and adventitia was scored by using a simplified scoring system of 4 for no injury, 3 for mild damage of structural components, 2 for moderate injury, 1 for severe injury, and 0 for complete destruction of the structural component. Five separate tissue specimens were prepared for each animal. At least 6 separate areas from each specimen were reviewed and graded, for a total of 30 data points per animal. For comparison, mean scores with SDs were calculated for each layer of the aortic wall for both wild-type and kininogen-deficient animals and compared by \( t \) test.

### Statistical Studies

The differences between the groups were compared by using Student’s \( t \) test.

### Results

Blood was available for 4 of the 5 animals in each group. The Brown-Norway rats of the Katholiek strain had no detectable HK compared with the value (92 \( \pm \) 3.7%) for the Brown-Norway wild-type (Table 1), in agreement with previous reports.12 We then determined whether the limited endothelial denudation produced changes consistent with disseminated intravascular coagulation (DIC). We measured antithrombin, whose concentration decreases as it forms complexes with thrombin; factor V, which decreases subsequent to successive cleavage by thrombin and activated protein C; and fibrinogen, which is depleted as it is converted to fibrin by thrombin. The concentrations of antithrombin, factor V, and fibrinogen were within the normal range for human plasma, and more important, there were no significant differences between the wild-type and kininogen-deficient rats (Table 1). The FDP levels, which reflect fibrinogen or fibrin proteolysis predominantly by plasmin, were at the upper limit of the normal values for human plasma, but there was no significant difference between the wild-type and kininogen-deficient rats. Thus, there was no evidence for DIC or diminished fibrinolytic response between the 2 groups of rats.

At the time of retrieval of the aortic specimens, no clot was found in 3 of the 5 wild-type rats, and a small amount of normal-appearing, mixed platelet-fibrin thrombus was seen in the remaining 2 animals. In the kininogen-deficient animals, mixed thrombus filled the entire injured segment.

### Blood Coagulation and Fibrinolytic Studies

Blood was drawn for coagulation studies into a plastic tube containing 3.8% sodium citrate (9:1, vol/vol). The samples were centrifuged at 4°C for 15 minutes at 3000g to obtain plasma for antithrombin, fibrinogen, factor V, and HK determinations. For the determination of serum fibrinogen degradation products (FDPs), blood was centrifuged, thrombin (25 NIH units/mL, Chromogenix) and aprotinin (final concentration, 200 U/mL; Miles, Inc) were added, and then the clotted serum was incubated at 37°C for 2 hours and centrifuged at 4°C for 15 minutes at 2500g. Both plasma and serum were frozen at \(-70^\circ \)C before the assays were performed. Antithrombin was measured by a functional microplate assay according to the method described by Scott13 and using the Coatest antithrombin kit (Pharmacia Hepar). The assay measures the ability of plasma to inhibit thrombin, as judged by hydrolysis of the tripeptide chromogenic substrate S-2238. Fibrinogen quantitation was measured in a fibrometer (BBL Fibrosystem) according to the modified thrombin time described by Claus.14 Factor V functional concentration was determined by coagulant assay15 with the use of a fibrometer (BBL Fibrosystem) and plasma samples from subjects with a hereditary deficiency of factor V (Sigma Diagnostics). The results were expressed as a percentage of pooled normal plasma. HK activity was evaluated according to a modification of the partial thromboplastin time assay described by Proctor and Rapaport16 and the use of total human kininogen-deficient plasma.2 FDPs were measured with the staphylococcal clumping test (Sigma Diagnostics) as described by Hawiger et al.17 The assay is nonimmunological and cross-reacts with fibrinogen and FDPs from most species, including the rat. Results were expressed as fibrinogen equivalents in micrograms per milliliter.

### Table 1. Coagulation and Fibrinolytic Determinations in Brown-Norway Rats

<table>
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<th>Katholiek Strain</th>
<th>Wild Type</th>
<th>Significance</th>
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<tr>
<td>HK, %*</td>
<td>0 ± 0</td>
<td>92 ± 3.7</td>
<td>( P &lt; 0.01 )</td>
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<tr>
<td>Antithrombin, %*</td>
<td>94 ± 1.2</td>
<td>98 ± 2.3</td>
<td>NS</td>
</tr>
<tr>
<td>Factor V, %*</td>
<td>89 ± 3.5</td>
<td>101 ± 3.5</td>
<td>NS</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL†</td>
<td>265 ± 6.5</td>
<td>288 ± 7.5</td>
<td>NS</td>
</tr>
<tr>
<td>FDPs, µg/mL‡</td>
<td>5.6 ± 0.95</td>
<td>4.6 ± 0.8</td>
<td>NS</td>
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*Results are expressed as mean \( \pm \) SEM of 4 animals, with a pool of 20 normal human plasma samples set equal to 100% in each of these 3 assays. The range of normal was 60% to 140% for HK and factor V and 90% to 110% for antithrombin.

†Human fibrinogen was 250 ± 11.1 mg/dL (mean \( \pm \)SEM) in the pool of 20 normals.

‡Normal range for FDPs in humans is 1.2 to 5.0 \( \mu \)g/mL.

#### Histological Studies

The histology of the midportion of the injured segment of the aortic specimens was reviewed by light microscopy for overall architecture and severity of injury by an observer blinded to the strain of the rat evaluated. Injury severity to the intima, media, and adventitia was scored by using a simplified scoring system of 4 for no injury, 3 for mild damage of structural components, 2 for moderate injury, 1 for severe injury, and 0 for complete destruction of the structural component. Five separate tissue specimens were prepared for each animal. At least 6 separate areas from each specimen were reviewed and graded, for a total of 30 data points per animal. For comparison, mean scores with SDs were calculated for each layer of the aortic wall for both wild-type and kininogen-deficient animals and compared by \( t \) test.

#### Statistical Studies

The differences between the groups were compared by using Student’s \( t \) test.
Review of the histological sections revealed a minor arterial wall injury (Figure 1A and 1B). The endothelium and internal elastic membrane were most frequently disrupted (Figure 2A and 2B). The elastic lamellae and circular smooth muscle layers of the inner portion of the media were also injured, but to a lesser extent. The outer portion of the media and the adventitia were uninjured. Smooth muscle cellular morphology within the media appeared normal. There was no hemorrhage or inflammatory cell infiltrate evident within the arterial wall. The fibrin stain revealed minimal fibrin in the kininogen-deficient rats (Figure 3A) but not in the wild-type rats (Figure 3B). Platelets were adherent to the subendothelium of the vessels (Figure 3A and 3B). Scoring of the injury to each layer of the arterial wall confirmed these findings (Table 2) and showed no significant difference in the degree of vascular injury between the wild-type and kininogen-deficient rats.

The time to 90% occlusion (Figure 4) varied from 110 to >240 minutes (mean±SEM, 194±29 minutes) for the wild-type Brown-Norway rats. In fact, 3 of the 5 showed no occlusion even at 240 minutes. In the kininogen-deficient rats, a 90% flow decrease was found at 7 to 95 minutes (38.4±17minutes). All animals in this group experienced thrombotic occlusion, and the differences versus the wild type were highly significant (P<0.002), with no overlap in the times to 90% flow reduction. Kinogen thus protected the wild-type rats from thrombosis in this minimal-injury model.

Discussion

The model used in this study to assess the thrombotic impact of kininogen deficiency is one of a mild, flow-surface injury. Commonly used models, such as Folt’s model, which creates a severe transmural arterial wall injury in conjunction with a hemodynamically significant stenosis, can induce rapid arterial thrombosis in normal animals. That model, though appropriate for the evaluation of antithrombotic research, is unsuitable for the detection of an enhanced thrombotic state. Therefore, the model developed for this study produced endothelial damage without severe underlying structural damage or the creation of a hemodynamically significant proximal stenosis. This mild, localized flow-surface alteration more closely imitates the forms of vascular injury experienced in patients and provides an excellent method for the detection of increased thrombus formation. The hypothesis was confirmed by the lack of thrombosis within the wild-type animals. The rapid onset of aortic thrombosis experienced by the kininogen-deficient animals suggests a strongly prothrombotic state, which can be triggered by even a mild flow-surface injury.

Models of limited arterial wall injury have been well described and usually result in the formation of mixed platelet-fibrin thrombi. The model developed herein has fulfilled our criteria in that there was limited intimal damage,
little medial injury, and no adventitial changes on histological examination. Moreover, the extent of damage in both rat strains was essentially identical, and thus, the lack of kininogen, confirmed by functional analysis, did not decrease or enhance the vascular injury. The limited nature of the coagulation changes induced was underlined by the fact that the usual plasma changes accompanying DIC, decreased factor V, fibrinogen, and antithrombin and elevated FDPs, did not occur, and that no significant differences were found between the 2 groups. The significant finding that the kininogen-deficient rats developed a 90% reduction in blood flow by 38 minutes, whereas the wild-type Brown-Norway rats showed this degree of flow reduction by 194 minutes, indicates that kininogen is functioning in normal rats to modulate blood changes that induce thrombosis.

The mechanism of this effect must reflect the functions of 1 or both kininogens. The heavy chain– and bradykinin-containing domains are common to both HK and LK. Kininogens (D2 and D3) are the major inhibitors of cysteine proteases in the extracellular milieu. Calpain, a calcium-activated intracellular cysteine protease, is inhibited by HK and LK, with a Ki equal to 2 and 0.5 nmol/L, respectively. Although calpain is present in the cytosol in resting platelets, when they are activated, calpain translocates to the external membrane, where it can be inhibited by kininogen. Although calpain inhibition can modulate platelet aggregation, it cannot prevent the earliest phases of platelet activation, because that effect requires the prior translocation. Thus, this action of the kininogens could potentiate the antithrombotic action but probably is not the major contributor.

The profibrinolytic properties of HK should next be considered. We have shown that HK binds to the urokinase receptor (uPAR) on endothelial cells through D2 and D3 of the uPAR. Because HK circulates in a complex with prekallikrein, HK binding to uPAR allows prekallikrein, which is activated by an endothelial protease, to be converted to kallikrein. The cleavage of prourokinase bound to D1 of uPAR by kallikrein can then proceed very efficiently. The dependence on the HK-prekallikrein interaction was established by the ability of a peptide from D6 of HK to inhibit plasmin formation. Cell-mediated fibrinolysis involving urokinase is thought to be more important for cell migration and invasion than is fibrin clot dissolution. However, bradykinin liberated by kallikrein cleavage of kininogen releases tissue plasminogen activator from endothelial cells, thereby enhancing fibrin clot lysis. Bradykinin, by its vasodilatory action, promotes local blood flow, minimizing stasis and enhancing fibrinolysis. Thus, in the absence of kininogen, fibrinolysis would be markedly reduced, limiting the dissolution of the fibrin clot and enhancing thrombus formation.

Finally, the ability of kininogen to inhibit thrombin binding to platelets and shift the dose-response curve 10-fold makes this effect a strong candidate for the antithrombotic effect of kininogen. The first 5 amino acids of bradykinin, a natural metabolite of the peptide, have been shown to inhibit cleavage of the heptaspanning G protein–coupled thrombin receptor and, thus, prevent platelet signal transduction by thrombin. Recently, we and others have shown that HK binds to the glycoprotein Ib/IX complex. Our studies indicate that HK inhibition of the binding of thrombin to platelets accounts for the requirement of greater thrombin concentrations for platelet activation by the heptaspanning thrombin receptor. Conversely, one expects kininogen to be protective at low thrombin concentrations, as in the model used in this study.

### TABLE 2. Injury Severity Scores

<table>
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<tr>
<th>Animal Type</th>
<th>Aortic Wall Layer</th>
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<tbody>
<tr>
<td></td>
<td>Intima*</td>
<td>Media*</td>
<td>Adventitia*</td>
</tr>
<tr>
<td>Wild type</td>
<td>2.56±0.51</td>
<td>2.96±0.85</td>
<td>3.44±0.70</td>
</tr>
<tr>
<td>Kininogen deficient</td>
<td>2.42±0.78</td>
<td>3.29±0.75</td>
<td>3.79±0.41</td>
</tr>
</tbody>
</table>

*P* > 0.05 for all 3 comparisons between wild-type and kininogen-deficient rats.
Clinical studies suggest that proteins in the contact system may have antithrombotic properties. Both prekallikrein and HK deficiencies are too rare to allow us to draw firm conclusions. However, there is an increased incidence of thrombosis in patients with congenital homozygous factor XII deficiency33–37 and an increased incidence of congenital factor XII deficiency in patients with venous thrombosis and acquired thrombotic disorders such as myocardial infarction38 and restenosis of common arteries after thrombolytic therapy.39

The clinical relevance of kininogen deficiency is as yet undefined. The results of this study suggest that severe kininogen deficiency present at birth might lead to the early appearance of significant thrombotic events. These might require conditions producing endothelial damage and, therefore, the deficiency is a risk factor, as are deficiencies in the protein C system or antithrombin. There are, however, many thrombotic events in neonates whose causes remain unexplained.40,41 Unfortunately, no assessment of kininogen has been attempted in these infants, which would determine the prevalence and impact of kininogen deficiency in early thrombotic events.

Because the thrombus in this animal model is formed in the aorta, platelet activation, thrombin production, and fibrin formation are likely events. Kininogens have the potential to enhance the dissolution of the fibrin clot. Because the effect on thrombin binding to platelets is mediated by D3, an LK deficiency also would be expected to contribute. Detailed studies using radioactively triggered platelets and fibrinogens are planned to elucidate the relative importance of these mechanisms in the antithrombotic effect of kininogens. Peptides inhibiting thrombin-induced platelet activation have been elucidated,5,30 and could be lead compounds for developing antithrombotic drugs.

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References


