Homocysteine, an Atherogenic Stimulus, Reduces Protein C Activation by Arterial and Venous Endothelial Cells

By George M. Rodgers and Marion T. Conn

Elevated blood levels of homocysteine are associated with atherosclerosis and thrombotic disease. We previously reported that treatment of cultured endothelial cells with homocysteine increased endogenous factor V activity by activation of the cofactor. Because endothelial cell-associated factor Va would be regulated by the protein C mechanism, the ability of homocysteine-treated arterial and venous endothelial cells to activate protein C was investigated. Both arterial and venous endothelial cells activated protein C: 0.6 mmol/L homocysteine reduced endothelial cell protein C activation by 12%. Maximal inhibition (90%) of protein C activation occurred with 7.5 to 10 mmol/L homocysteine after 6 to 9 hours of incubation. Metabolism of homocysteine was not accelerated by cultured endothelial cells. Investigation of the mechanism(s) by which homocysteine reduced protein C activation indicated that the metabolite did not induce an inhibitor to activated protein C, but in low concentrations acted as a competitive inhibitor to thrombin. These data suggest that perturbation of the vascular endothelial cell protein C mechanism by homocysteine may contribute to the thrombotic tendency seen in patients with elevated blood levels of this metabolite.

ENDOTHELIAL CELLS actively maintain vessel wall thromboresistance by expressing several antithrombotic properties, including tissue plasminogen activator, prostacyclin, heparin-like glycosaminoglycans, and thrombomodulin (TM). TM is an integral endothelial cell membrane protein involved in the protein C pathway of coagulation. The importance of the protein C pathway is emphasized by the finding that patients with an inherited deficiency of protein C have a congenital thrombotic disorder. Abnormalities in TM activity may also be important in predisposing to a thrombotic tendency; perturbation of vascular cells by monokines in vitro and in vivo has been reported to suppress TM activity, resulting in a net expression of vascular prothrombotic properties. In addition to these immunologic stimuli, endotoxin also suppresses endothelial cell TM activity; this effect might contribute to intravascular coagulation associated with gram-negative sepsis.

The mechanisms by which atherogenic stimuli regulate endothelial cell coagulant properties have not been well-defined. We have focused on the synthesis and activation of factor V, the cofactor required for optimal Factor Xa-catalyzed prothrombin activation. Arterial vascular endothelial cells synthesize factor V, and arterial and venous endothelial cells express this activity on their luminal surface. Homocysteine, the atherogenic sulfur-containing amino acid, enhanced endothelial cell factor V activity by inducing a protease that activates (cleaves) the procofactor to factor Va. The association of elevated blood levels of homocysteine with a congenital thrombotic disorder suggested that enhanced vascular factor V activity might contribute to this hypercoagulable state. Whether or not homocysteine-treated cells also exhibit diminished TM activity has not been determined; decreased TM activity would result in failure to inactivate factor Va and increased expression of vessel wall procoagulant activity.

The present study was designed to determine if homocysteine-treated endothelial cells exhibit reduced TM activity, and if so, to determine the mechanism(s) by which homocysteine reduces TM activity.

METHODS

Homocysteine, cysteine, methionine, hirudin, catalase, Trizma base, and porcine heparin were obtained from Sigma Chemical Co (St Louis, MO). The chromogenic substrate S-2238 was supplied by Helena Laboratories (Beamont, TX). Sodium [113]Cr] chromate (specific activity 200 Ci/g) was provided by New England Nuclear Corp (Boston, MA). Medium for cell culture was obtained from the University of California, San Francisco Cell Culture Facility, and contained less than 10 pg/mL endotoxin. Fetal calf serum and calf serum were provided by HyClone (Logan, UT) and contained less than 13 pg/mL endotoxin. Purified human protein C, activated protein C (APC), protein S, and bovine thrombin and protein C were supplied by Enzyme Research Laboratories (South Bend, IN). Purified human α-thrombin (specific activity 4,000 U/mL) was supplied by Dr John W. Fenton II (New York State Department of Health, Albany, NY).

Cell culture techniques. Human umbilical vein endothelial (HUVE) cells were obtained as described and were used between passages 2 and 3. Both fibroblast growth factor and porcine heparin were added to cultured HUVE cells. Assays were performed on cells plated in 35 mm Petri dishes or in 24-well trays. Adult bovine aortic endothelial (ABAE) and bovine pulmonary vein endothelial (BPVE) cells were cultured as described using fibroblast growth factor and were used between passages 5 and 9.

Homocysteine treatment of cultured cells and APC assay. Homocysteine was dissolved in serum-containing medium at the desired concentration (0.3 to 10 mmol/L), sterile-filtered, and added to cell monolayers 1 to 2 days postconfluence. Most incubations were performed for 5 to 6 hours in the tissue culture incubator. Endothelial cell activation of protein C was measured using a previously described method.

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described amidolytic assay. After incubation with control or homocysteine-containing media, cell monolayers were washed three times with serum-free medium, and the following reagents were added for routine assay of TM activity: thrombin (0.1 U/mL), protein C (12 μg/mL). After a 1-hour incubation at 37°C, thrombin activity was neutralized with excess hirudin, and APC activity was quantitated using the chromogenic substrate S-2238. Thrombomodulin activity was obtained from a standard curve constructed using purified APC in the standard assay. Results are expressed as APC activity in ng/mL/min. This amidolytic assay was linear between 50 and 4,000 ng/mL. APC. Omission of endothelial cells, thrombin, or protein C resulted in less than 2% of the amidolytic activity measured when all three components were assayed. Cell viability was greater than 95% as determined by trypan blue staining and [3H] release.

**Conversion of homocysteine to homocysteine by cultured endothelial cells.** A Beckman Model 119 CL Amino Acid Analyzer (Palo Alto, CA) was used to quantitate conversion of homocysteine to homocysteine by HUVE cells. HUVE media containing 10 mmol/L homocysteine was incubated either in empty Petri dishes or Petri dishes containing confluent HUVE cells. At intervals from 0 to 24 hours, conditioned media was obtained and diluted 1:4 with 10% 5-sulfosalicylic acid. Precipitated protein was removed by centrifugation, and the supernatant was diluted 1:100 in 0.1 N HCl before amino acid analysis. Samples of 100 μL volume (containing ~10 nmol/L homocysteine) were loaded onto the analyzer, as were standard concentrations of homocysteine and homocystine. Oxidation of homocysteine to homocysteine was quantitated using standard curves relating peak height with known concentrations of pure amino acids.

**Kinetic analysis.** Kinetic analysis of thrombomodulin activity was assisted by a nonlinear least squares procedure using a Hewlett-Packard 9825 computer (Palo Alto, CA). These experiments were performed in duplicate. Thrombin alone rates were not subtracted at each thrombin concentration. The results of analyses are presented in terms of the mean parameter values ± estimates of standard errors obtained from the nonlinear least squares analysis. Data were analyzed with models of increasing complexity. The model that was chosen for reporting the results is that which yielded the smallest sum of squares, and which represented a significant improvement over simpler models. Thus, in the best model, the experimental data were fitted by a hyperbola with additional terms: V = V_{max}S/(km + S) + aS, where V is the velocity of the reaction (activation of protein C), V_{max} and V_{max} are maximal velocities of two states, and a is the slope of the linear increase over the saturation characterized by V_{max}. Finally, S is the substrate concentration, and Km is the corresponding Michaelis-Menten equilibrium constant.

**RESULTS**

Because homocysteine regulation of endothelial cell factor V activity was demonstrated both with bovine aortic cells and HUVE cells, experiments were conducted to determine whether cultured arterial and venous endothelial cells could activate protein C and whether homocysteine-treated cells exhibited reduced TM activity. Bovine cell lines (ABAE, BPVE) were studied since human arterial cells were not routinely available. Bovine protein C (12 μg/mL) and bovine thrombin (0.1 U/mL) were used in these studies, and homocysteine was tested at a concentration of 10 mmol/L, a concentration previously found to have maximal effects on enhancing endothelial cell factor V activity. The data presented in Table 1 indicate that both cultured bovine arterial and venous endothelial cells activated protein C, and that homocysteine treatment reduced generation of APC by 53% and 50%, respectively. In other experiments, the requirement for serum in the homocysteine reduction in TM activity was studied. In contrast to the previous report that described a serum requirement for endotoxin reduction of TM activity, the homocysteine effect did not require the presence of serum (data not shown).

Subsequent studies were performed with HUVE cells and human proteins, allowing a comparison of the effects of homocysteine with previous studies on TM activity. Figure 1 depicts a time-course study of APC generation by homocysteine-treated (10 mmol/L) HUVE cells. Within 30 minutes, greater than 50% reduction in APC generation occurred, with maximal reduction (10% to 20% of initial activity) observed after 6 hours. Recovery to ~60% original TM activity occurred after 24 hours. A time course study of APC generation by ABAE cells (using bovine proteins) yielded similar results (data not shown).

Figure 2 summarizes data from dose-response experiments of homocysteine on HUVE cell TM activity. Exposure of cultured cells to homocysteine concentrations as low as 0.6 to 1.25 mmol/L for 6 hours resulted in consistent reductions in APC activity of 12% and 33%, respectively. Maximal reduction in TM activity (90%) was seen with 7.5 to 10 mmol/L homocysteine. Regulation of TM activity by homocysteine occurred at metabolite concentrations reported to enhance endothelial cell factor V activity.

To determine the selectivity of the homocysteine effect on endothelial cell TM activity, other sulfur-containing amino acids were assayed in a similar manner. Methionine and

<table>
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<tr>
<th>Table 1. Comparison of Protein C Activation by Bovine Arterial and Venous Endothelial Cells</th>
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<tr>
<td><strong>Medium</strong></td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>H</td>
</tr>
<tr>
<td>C 2,500</td>
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<tr>
<td>H 1,333</td>
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</table>

ABAE or BPVE cells were incubated with control (C) or 10 mmol/L homocysteine (H)-containing medium for 12 hours. Endothelial cell activation of protein C was then measured using bovine protein C (12 μg/mL) and bovine thrombin (0.1 U/mL) in an amidolytic assay (see Methods).

[Figure 1. Time-course study of APC generation by homocysteine-treated HUVE cells. Confluent HUVE cells were incubated with 10 mmol/L homocysteine for varying times. APC activity was determined using an amidolytic assay (see Methods). Results are expressed as percent reduction in initial APC activity of control HUVE cells. Each value represents the average of two experiments.]
cysteine were compared with homocysteine at equimolar concentrations (10 mmol/L). In these experiments, homocysteine-treated cells exhibited $\sim$80% reduction in TM activity. In contrast, TM activity of methionine- or cysteine-treated cells was reduced only $\sim$20%.

Starkbaum and Harlan reported that homocysteine induces endothelial cell injury by generation of hydrogen peroxide. To determine whether the homocysteine-induced reduction in endothelial cell TM activity might be related to hydrogen peroxide generation, catalase (1,000 U/mL) was incubated with endothelial cells treated with either control media or media containing 5 mmol/L or 10 mmol/L homocysteine. After 5 hours of incubation, TM activity was assayed. Coincubation of homocysteine-treated cells with catalase did not prevent the reduction in TM activity.

To determine if oxidation of homocysteine to homocystine by cultured cells correlated with the data obtained in the time-course study (Fig 1) or the dose-response curve (Fig 2), we measured homocysteine concentrations using an amino acid analyzer. The concentration of homocysteine in media incubated with endothelial cells or with an empty plastic dish was observed to fall slowly with time, and correlated with rising homocystine levels. Within 1 to 2 hours, the homocysteine concentration fell 5% to 10%. By 6 to 8 hours, 25% homocysteine was oxidized, and after 24 hours, 57% was oxidized. Nonlinear least squares analysis of these data indicated no significant difference in the rate of disappearance of homocysteine or rate of appearance of homocystine between empty Petri dishes and dishes containing confluent endothelial cells. These data indicate that cultured normal endothelial cells do not enhance the oxidation of homocysteine. These data may explain recovery of TM activity observed after 24 hours of treatment, since the initial concentration of homocysteine in culture media was reduced by greater than 50% after 24 hours. Since most of our experiments measured TM activity after 5- to 6-hour exposure to homocysteine, oxidation of this amino acid by endothelial cells was not a major factor in these studies. These data also suggest that the sulfur amino acid primarily responsible for the effects seen on TM activity in our model is homocysteine, and not homocystine.

As has been previously noted, several mechanisms could be responsible for the diminished TM activity of perturbed endothelial cells, including cellular expression or secretion of an inhibitor to APC, reduction in the amount of TM expressed, or reduced affinity for protein C or thrombin. The issue of whether homocysteine-treated endothelial cells secrete an APC inhibitor was addressed by incubating confluent HUVE cells with control media or media containing 10 mmol/L homocysteine for 6 hours, washing the cells three times with serum-free medium, and then incubating the cells with serum-free medium for 1 hour. Purified human APC (1.5 μg/mL) was added to each sample of conditioned medium and incubated for an additional 1 hour. These samples were then assayed for APC amidolytic activity. No inhibition of APC activity occurred with the conditioned medium obtained from homocysteine-treated endothelial cells. The possible expression of a homocysteine-induced, cell-associated APC inhibitor was studied by incubating purified human APC (1.5 μg/mL) for 1 hour with control or homocysteine-treated HUVE cells, in the presence or absence of human protein S (18 μg/mL). No reduction in APC amidolytic activity was observed with homocysteine-treated HUVE cells. Thus, cellular expression or secretion of an APC inhibitor does not explain the reduction in TM activity after homocysteine treatment.

The kinetic effects of homocysteine on endothelial cell activation of protein C were investigated. The dissociation constant ($k_d$) for thrombin was determined with control or homocysteine-treated HUVE cells, using thrombin concentrations ranging from 0.05 to 10 nmol/L. The $k_d$ for thrombin using untreated HUVE cells was $0.17 \pm 0.04$ nmol/L, a value similar to that previously reported. Table 2 summarizes the $k_d$ and $V_{max}$ data from these experiments. At homocysteine concentrations of 1, 2, 4, and 10 mmol/L, the $k_d$ values for thrombin were $0.19 \pm 0.06$ nmol/L, $0.54 \pm 0.21$ nmol/L, $1.14 \pm 0.12$ nmol/L, and $2.49 \pm 1.12$ nmol/L, re-

### Table 2. Effect of Homocysteine Concentration on the $k_d$ and $V_{max}$ for Thrombin

<table>
<thead>
<tr>
<th>Homocysteine (mmol/L)</th>
<th>$k_d$ (nmol/L)</th>
<th>$V_{max}$ (ng/mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$0.17 \pm 0.04$</td>
<td>$4.570 \pm 35$</td>
</tr>
<tr>
<td>1</td>
<td>$0.19 \pm 0.06$</td>
<td>$4.182 \pm 418$</td>
</tr>
<tr>
<td>2</td>
<td>$0.54 \pm 0.21$</td>
<td>$4.730 \pm 851$</td>
</tr>
<tr>
<td>4</td>
<td>$1.14 \pm 0.12$</td>
<td>$4.670 \pm 446$</td>
</tr>
<tr>
<td>10</td>
<td>$2.49 \pm 1.14$</td>
<td>$1.485 \pm 204$</td>
</tr>
</tbody>
</table>

HUVE cells were incubated with control media or homocysteine-containing media at the indicated concentrations for 6 hours, then washed with serum-free medium. Thrombin (0.05 to 10 nmol/L) and protein C (1 μmol/L) were added, and APC activity was determined using an amidolytic assay (see Methods). The data were analyzed by a nonlinear, least squares procedure, and the $k_d$ and $V_{max}$ values $\pm$ SE were obtained for each homocysteine concentration.
spectively. Figure 3 summarizes results of these experiments using untreated cells, cells treated with 2 mmol/L homocysteine, and cells treated with 10 mmol/L homocysteine.

The kinetic analysis of the thrombin-thrombomodulin interaction used the correction factors, $V_0$ and $a$, in order to obtain unbiased estimates of $V_{\text{max}}$ and $k_d$. We observed that for homocysteine concentrations less than 4 mmol/L, $V_0$ was too small to be determined, and at the 10 mmol/L concentration, the value of $a$ was negligible. The value of $V_{\text{max}}$ was independent of homocysteine concentration except at the 10 mmol/L concentration, when $V_{\text{max}}$ was reduced by half. Thus, with respect to thrombin kinetics, diminished protein C concentrations ranging from 20 to 5,000 nmol/L, $V_0$ and $V_{\text{max}}$ values obtained using untreated cells and cells treated with 2, 4, and 10 mmol/L homocysteine. The $k_m$ for protein C on control cells was $1.54 \pm 0.26$ μmol/L, a value similar to that previously reported.6 The effects of homocysteine on protein C kinetics were less substantial than those seen with thrombin kinetics.

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In other experiments, the $k_m$ for protein C was determined with control or homocysteine-treated HUVE cells, using protein C concentrations ranging from 20 to 5,000 nmol/L. Table 3 summarizes the $k_m$ and $V_{\text{max}}$ values obtained using untreated cells and cells treated with 2, 4, and 10 mmol/L homocysteine. The $k_m$ for protein C on control cells was $1.54 \pm 0.26$ μmol/L, a value similar to that previously reported.6 The effects of homocysteine on protein C kinetics were less substantial than those seen with thrombin kinetics.

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A corollary of these data indicating that homocysteine is a competitive inhibitor to thrombin is that thrombin might be predicted to limit homocysteine inhibition of protein C activation when included in the initial incubation mixture. This hypothesis was tested by coincubating HUVE cells with thrombin (144 to 720 nmol/L) and 2.5 and 4 mmol/L homocysteine for 5 hours before measurement of protein C activation. As summarized in Table 4, homocysteine treatment alone reduced protein C activation compared with untreated cells. Coincubation of thrombin with homocysteine attenuated the homocysteine inhibition at each homocysteine concentration. No such enhanced effect on protein C activa-

Table 3. Effect of Homocysteine Concentration on the km for Protein C

<table>
<thead>
<tr>
<th>Homocysteine (mmol/L)</th>
<th>$k_m$ (μmol/L)</th>
<th>$V_{\text{max}}$ (ng/mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.54 ± 0.26</td>
<td>6,007 ± 540</td>
</tr>
<tr>
<td>2</td>
<td>1.20 ± 0.10</td>
<td>6,049 ± 201</td>
</tr>
<tr>
<td>4</td>
<td>2.10 ± 0.53</td>
<td>6,800 ± 168</td>
</tr>
<tr>
<td>10</td>
<td>6.73 ± 2.17</td>
<td>2,055 ± 719</td>
</tr>
</tbody>
</table>

HUVE cells were incubated with control media or homocysteine-containing media at the indicated concentrations for 6 hours, then washed with serum-free medium. Protein C (20 to 5,000 nmol/L) and thrombin (1 nmol/L) were added, and APC activity was determined using an amidolytic assay (see Methods). The data were analyzed by a nonlinear, least squares procedure, and the $k_m$ and $V_{\text{max}}$ values ± SE for each homocysteine concentration were obtained.
HOMOCYSTEINE REDUCES PROTEIN C ACTIVATION

...tions were exerted on untreated cells by similar concentrations of thrombin (Table 4). Higher concentrations of thrombin (≥ 1 μmol/L) induced gross changes in cell morphology and induced cell lysis of control and homocysteine-treated HUVE cells.

DISCUSSION

Homozgyous homocystinuria has long been recognized as a risk factor for thrombosis. Homocystinuria results most often from deficiency of cystathionine β-synthase, an enzyme necessary for transsulfuration. This genetic defect results in failure of conversion of homocysteine to cysteine, leading to accumulation in blood of sulfur-containing amino acids (homocysteine, homocystine, methionine). Recently, heterozygotes were reported to be predisposed to premature vascular disease, and elevated plasma levels of homocysteine were associated with peripheral arterial occlusive disease. Although we previously demonstrated that homocysteine-treated endothelial cells activated factor V by the induction of a protease, it has not been established whether treated cells exhibit reduced TM activity, and thus fail to inactivate factor Va. Activated protein C has been previously reported to inactivate endothelial cell-associated Factor Va.

Immunocytochemical studies have shown that TM antigen is widely distributed in a variety of human and rabbit vascular tissues. Although a comparison of functional TM activity of capillary and venous endothelial cells has been reported, data on the TM activity of arterial and venous vascular tissues are not available. Because homocysteine treatment of cultured arterial and venous endothelial cells results in enhanced factor V activity, we compared TM activity of these vascular cell types in the basal state, as well as after homocysteine treatment. Table 1 indicates that TM activities of bovine arterial and venous cells were roughly equivalent, and that homocysteine reduced TM activity of both cell types by ~50%. These data are consistent with clinical information that homocystinuric patients experience both venous and arterial thrombosis, and suggest that the protein C pathway may contribute to the thrombotic tendency in this disorder.

Table 4. Effect of Thrombin in Limiting the Homocysteine-Induced Reduction in Protein C Activation

<table>
<thead>
<tr>
<th>Thrombin Concentration (nmol/L)</th>
<th>H-144</th>
<th>H-288</th>
<th>H-430</th>
<th>H-720</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 2.5 mmol/L</td>
<td>168 (48%)</td>
<td>160 (47%)</td>
<td>202 (35%)</td>
<td>210 (36%)</td>
</tr>
<tr>
<td>H 4 mmol/L</td>
<td>80 (75%)</td>
<td>130 (57%)</td>
<td>170 (45%)</td>
<td>165 (50%)</td>
</tr>
</tbody>
</table>

Washed HUVE cells were incubated with buffer or thrombin at the indicated concentration without (C) or with homocysteine (H) for 5 hours before measurement of protein C activation. APC activity (ng/mL/min) of control and homocysteine-treated cells is depicted. The percent reduction in APC activity induced by homocysteine treatment with and without thrombin is shown in parenthesis. Data from duplicate dishes of a representative experiment are shown.

Whether bovine arterial cells possess more basal TM activity than bovine venous cells (as suggested in Table 1) is uncertain. In these studies, bovine venous cells expressed ~70% of the TM activity expressed by bovine arterial cells. Any differences between the coagulant properties of these two endothelial cell lines may reflect the differences in passage number. A previous report indicated that human capillary and venous endothelial cells also expressed similar amounts of TM activity. Thus, TM activity may be similar in arterial, venous, and capillary endothelial cells.

Although homocysteine treatment of both bovine and human endothelial cells resulted in a reduction in TM expression, HUVE cells exhibited a greater sensitivity to homocysteine than did bovine arterial or venous cells (90% reduction for HUVE cells [Fig 1] versus 50% reduction for bovine cells [Table 1]). The species difference may explain these results.

Our data indicating equivalent rates of oxidation of homocysteine to homocystine by cellular and noncellular surfaces suggest that normal endothelial cells do not promote homocysteine oxidation. Endothelial cells derived from a patient with homocystinuria would be expected to exhibit different properties with respect to homocysteine metabolism, but these studies have not yet been performed. De Groot et al studied HUVE cells derived from an obligate heterozygote for homocystinuria. These investigators observed increased 35Cr release from prelabeled cultured endothelial cells derived from the heterozygote compared with cells derived from a normal individual when cells were incubated with methionine and homocysteine, but not homocystine. The metabolism of homocysteine by normal or heterozygote endothelial cells was not reported in this study.

A comparison of the time course of homocysteine-induced suppression of TM activity with that reported after perturbation by tumor necrosis factor (TNF), interleukin-1 (IL-1), and endotoxin indicates that the effect of homocysteine is most similar to that induced by the monokines. Both TNF and IL-1 reduced endothelial cell TM activity within 1 hour of treatment, with maximal effects seen after ~6 hours, associated with a 70% to 80% reduction of original TM activity. This is similar to results obtained with homocysteine-treated cells in the present study (Fig 1). In contrast, endotoxin suppression of TM activity occurred after 6 hours with peak effects observed after 24 hours, associated with ~50% reduction of TM activity. Maximal suppression of TM activity by homocysteine occurred at a time when enhanced endothelial cell factor Va activity was seen, 6 to 9 hours after homocysteine treatment. Thus, at least in vitro, homocysteine-induced endothelial cell procoagulant activity would be amplified by diminished TM activity.

There are several mechanisms by which a sulfur-containing amino acid like homocysteine might exert an effect on endothelial cell function. First, the effect might be dependent...
on the free thiol group. This is less likely since other related amino acids with free thiol groups (methionine, cysteine) were not active in inhibiting endothelial cell TM activity. A second possibility is that the effect might relate to generation of hydrogen peroxide, which could oxidize endothelial cell membrane proteins, including TM. Incubation of catalase with homocysteine-treated cells, however, did not prevent the reduction in TM activity, suggesting that hydrogen peroxide formation may not be important in this response. A third possibility is that homocysteine induces an endothelial cell inhibitor to APC, but we were unable to demonstrate secreted or cell-associated inhibitors. A fourth possibility is that homocysteine may directly interact with TM to impair TM-thrombin or TM-protein C interactions. We observed that homocysteine-treated cells exhibited reduced affinity for thrombin.

Kinetic analysis of the thrombin experiments indicated that the $k_{cat}$ was a linear function of homocysteine concentration (Fig 4), suggesting that homocysteine acted as a competitive inhibitor to thrombin. The effects of homocysteine on the $K_m$ for protein C were less dramatic than the effects of the metabolite on thrombin affinity (Table 2). Given the low concentrations of homocysteine present in homocystinuric patients (less than 1 mmol/L), our data suggest that, if homocysteine affects the kinetics of TM in vivo, the metabolite will act primarily as a competitive inhibitor to thrombin. This hypothesis is supported by the data in Table 4 that indicate that thrombin can attenuate the effect of homocysteine in reducing protein C activation. However, since thrombin can exert multiple effects on endothelial cell hemostatic properties, there may be alternative explanations for the data in Table 4. Future studies with purified TM or its fragments may provide additional support for our observations.

Competitive inhibition of the thrombin-TM interaction by homocysteine does not completely explain the reduced TM activity seen with homocysteine-treated cells. If competitive inhibition were the sole mechanism of the homocysteine effect, it might be expected that the dose-response studies would parallel the kinetic studies, with concentrations of homocysteine $\leq 0.5$ mmol/L, reducing generation of APC activity by 50%. In fact, the concentration of homocysteine required to reduce TM activity by 50% was observed to be fivefold higher, $\sim 2.5$ mmol/L (Fig 2). Additionally, if competitive inhibition were the only mechanism for the homocysteine effect, excess thrombin would be predicted to completely antagonize the homocysteine effect. However, as indicated in Table 4, attenuation of the homocysteine effect by thrombin was incomplete. These data suggest that additional mechanisms may contribute to the homocysteine effect.

Other possibilities for homocysteine regulation of TM activity that we have not investigated include enhanced turnover (endocytosis) of TM$^{21-23}$ and reduction of TM mRNA in treated cells.$^{22}$ Recently, TM activity and mRNA half-life was measured in endothelial cells treated with phorbol ester.$^{23}$ This stimulus decreased TM functional activity and reduced surface antigen expression, without reducing TM mRNA half-life.$^{23}$ Moore et al reported that TNF also induced internalization and degradation of TM.$^{22}$ In both studies, TM surface activity was reduced by 50% with TNF or phorbol ester after 6 to 8 hours and $\sim 10$ hours of treatment, respectively. Whether the rapid effect of homocysteine on reducing endothelial cell TM activity ($1/2 \sim 30$ minutes) results from enhanced endocytosis is unknown.

Although the mechanisms responsible for reduced TM activity have not been completely defined, these data support the hypothesis that the endothelial cell protein C mechanism can be perturbed by an atherogenic amino acid. The previously described increased vascular procoagulant activity associated with homocysteine treatment,$^{10}$ coupled with the present observation that homocysteine competitively inhibits the thrombin-TM interaction, indicates that perturbation of vascular coagulant mechanisms may contribute to the thrombotic tendency seen in patients with elevated blood levels of this metabolite.

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