Characterization of an Inducible Endothelial Cell Prothrombin Activator

By Lihua Liu and George M. Rodgers

In vivo prothrombin activation is thought to occur via a factor Xα/factor V-dependent mechanism. We investigated whether human venous endothelial cells (EC) could be induced to express a prothrombin activator. EC treated with lipopolysaccharide (LPS) or interleukin-1 activated prothrombin in the absence of exogenous factors Xα and V. This activity resided in the membrane fraction of EC and was not inhibited by an antibody to factor V. The apparent Km value was 3.3 ± 0.3 μmol/L. Comparative studies of thrombin generation using a model system of phospholipid and factors Xα/V versus LPS-treated EC were performed to quantify the effects of known inhibitors to factor Xα. The factor Xα inhibitor DEGR-chloromethyl ketone and an antibody to factor Xα inhibited prothrombin activation. However, the EC activator did not hydrolyze a factor Xα chromogenic substrate, and recombinant tick anticoagulant peptide did not suppress activity of the prothrombin activator. The apparent molecular weight of the EC activator was ~30 kDa. Exogenous factor V enhanced the activity of the EC activator, such that in the presence of factor V, the apparent Km value was 1.28 ± 0.10 μmol/L. Additionally, LPS-treated EC activated exogenous factor V. This activator has several characteristics of a previously described inducible murine monocyte prothrombin activator and may contribute to thrombin generation associated with pathologic stimuli.

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lysate activity was expressed as mU/dish. LPS-treated EC were incubated with TBS and prothrombin (140 μg/mL) with or without an antibody to factor V (100 μg/mL). Aliquots were removed at 5, 30, 60, and 120 minutes to quantitate thrombin generation.

Prothrombin was iodinated using the Iodogen method by incubating 0.5 mg protein at 4°C with 1 μCi of 125I in an Iodogen-coated Eppendorf tube. After 20 minutes, the radiolabeled prothrombin was separated from unincorporated iodine by using gel filtration chromatography. The specific activity of the radiolabeled protein ranged between 10 to 20 × 10^6 cpm/μg. A functional assay show that the activity of 125I-prothrombin was equivalent to that of unlabeled prothrombin.

Phospholipid vesicles were prepared by mixing 1 mg phosphatidylserine with 3 mg phosphatidylcholine in 1 mL TBS and sonicating for 30 minutes at 30% duty cycle. The sample was maintained in an ice bath under a nitrogen stream during this period. After sonication, liposomes were filtered using a 0.45-μm filter.

To determine whether certain protease inhibitors could affect activity of the EC activator, EC prothrombin activator activity was extracted from LPS-treated EC (1 mol/L NaCl, 30 minutes); extracts were subsequently dialyzed against TBS before the assay.

To determine whether protein synthesis was required for induction of the prothrombin activator, EC were pretreated with TBS or cycloheximide (10 μg/mL) 2 hours before addition of LPS. Thrombin generation was then measured as described above.

A comparison of 125I-prothrombin cleavage products generated by normal EC and factors Xa/V versus LPS-treated EC was performed. Aliquots of incubations were analyzed by reduced-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and autoradiography.

The molecular weight of the prothrombin activator was estimated using gel slicing and bioassay. LPS-treated EC (six 100-mm dishes) were extracted with high salt (1 mol/L NaCl). The extract was dialyzed against TBS, and then 100 μg/plate were applied to a 10% nonreduced SDS gel. The completed gel was sliced into 0.5-cm segments and eluted into TBS using a Centrifier apparatus. Eluates were then assayed for prothrombin activation and apparent molecular weight estimated for the biologically active fraction.

To determine whether LPS-treated EC could activate factor V, EC were incubated either with control media or LPS-containing media (1 μg/mL) for 4 hours, washed with M199, then incubated with M199 containing factor V (2 pg/mL) for 2 hours. Aliquots of incubations were analyzed by reduced-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and autoradiography.

The prothrombin activator was characterized using protease inhibitors and was compared in two model systems. In one model, the effects of factor Xa inhibitors on the activity of the inducible EC prothrombin activator were compared with control EC and classical prothrombinase (factors Xa/V) using equivalent EC surface areas. In a second model, the inducible EC prothrombin activator was compared with a standard system of phospholipid and factors Xa/V such that both generated equivalent amounts of thrombin. Table 1 summarizes results of both comparisons. Although factor Xa inhibitors such as dansyl-Glu-Gly-Arg chloromethyl ketone

RESULTS

In initial experiments, we observed that when LPS-treated EC were incubated with Medium 199 and prothrombin, thrombin generation occurred. No thrombin generation occurred with control EC and prothrombin. Optimal thrombin activation occurred with TBS (pH, 7.4), 5 mmol/L calcium, and 0.8 mmol/L magnesium. Maximal thrombin formation occurred with EC treated with LPS (1 μg/mL; 4 hours), or with IL-1β (10 U/mL; 4 hours). When LPS-treated EC were subsequently incubated with 140 μg/mL prothrombin, 5 mmol/L calcium, and 0.8 mmol/L magnesium, 6 mmol/L thrombin was generated after 2 hours. When LPS-EC lysates were subjected to ultracentrifugation (at 100,000g for 1 hour), 95% of the prothrombin activator activity resided in the membrane fraction.

Figure 1 shows a prothrombin concentration-response curve using the conditions listed above and EC grown in 24-well trays. The apparent Km was 3.3 ± 0.3 μmol/L, a value approximately threefold greater than the plasma concentration of prothrombin. Similar results were obtained when EC were induced with IL-1β (data not shown).

The prothrombin activator was characterized using protease inhibitors and was compared in two model systems. In one model, the effects of factor Xa inhibitors on the activity of the inducible EC prothrombin activator were compared with control EC and classical prothrombinase (factors Xa/V) using equivalent EC surface areas. In a second model, the inducible EC prothrombin activator was compared with a standard system of phospholipid and factors Xa/V such that both generated equivalent amounts of thrombin.
Table 1. Effect of Factor X\textsubscript{i} Inhibitors on Thrombin Generation by LPS-EC and Phospholipid Vesicles and Factors X\textsubscript{i}/V

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>LPS-EC</th>
<th>Control EC/X\textsubscript{i}/V</th>
<th>LPS-EC</th>
<th>PL/X\textsubscript{i}/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF (2 mmol/L)</td>
<td>86*</td>
<td>31</td>
<td>85*</td>
<td>21</td>
</tr>
<tr>
<td>DFP (10 mmol/L)</td>
<td>83*</td>
<td>99</td>
<td>89*</td>
<td>99</td>
</tr>
<tr>
<td>DEGR (10 \mu mol/L)</td>
<td>95</td>
<td>100</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Anti-V (100 \mu g/mL)</td>
<td>01</td>
<td>99</td>
<td>41</td>
<td>90</td>
</tr>
<tr>
<td>Anti-X (100 \mu g/mL)</td>
<td>97</td>
<td>94</td>
<td>97</td>
<td>99</td>
</tr>
<tr>
<td>r-TAP (100 nmol/L)</td>
<td>42</td>
<td>90</td>
<td>66</td>
<td>99</td>
</tr>
</tbody>
</table>

The results of two comparative studies are summarized. The left side compares the effects of factor X\textsubscript{i} inhibitors on thrombin generation in the LPS-EC model versus an equivalent surface area of control EC with factors X\textsubscript{i}/V. Reactant concentrations were as follows: LPS-EC experiment: factor V (6 \mu g/mL), prothrombin (140 \mu g/mL); control EC/X\textsubscript{i}/V: factor V (6 \mu g/mL), prothrombin (140 \mu g/mL), factor X\textsubscript{i} (20 ng/mL); PL/X\textsubscript{i}/V: factor V (6 \mu g/mL), prothrombin (140 \mu g/mL), factor X\textsubscript{i} (20 ng/mL), phospholipid vesicles [1:10,000 dilution of stock PL (1 mg PS:3 mg PC in 1 mL TBS)]. Results are expressed as the percentage of reduction from baseline thrombin generation. Baseline LPS-EC thrombin generation was 480 ± 217 nmol/L (n = 3), and baseline thrombin generation with control EC and factors X\textsubscript{i} and V was 865 ± 250 nmol/L (n = 3). The right side of the table compares the effects of factor X\textsubscript{i} inhibitors on thrombin generation in the LPS-EC model versus a model system of phospholipid (PL) vesicles and factors X\textsubscript{i}/V in which thrombin generation rates were similar (-900 nmol/L (n = 3)). Results shown are the mean of duplicate experiments.

* In this experiment, prothrombin activator activity was extracted from EC as described in Materials and Methods, treated with the protease inhibitor and subsequently incubated with prothrombin.
† This experiment was performed in the absence of exogenous Factor V.

and an antibody to factor X inhibited thrombin generation by LPS-EC and control EC/X\textsubscript{i}/V equivalently, r-TAP was less effective in inhibition of thrombin formation by LPS-EC. Similar inhibition results were observed when lower anti-factor X antibody concentrations were tested. A 10- \mu g/mL antibody concentration reduced LPS-EC thrombin formation by 29\%, while reducing control EC/X\textsubscript{i}/V thrombin formation by 11\%. At 25 \mu g/mL antibody concentration, LPS-EC was inhibited by 53\%, whereas control EC/X\textsubscript{i}/V was inhibited 45\%. At 50 \mu g/mL antibody concentration, LPS-EC was inhibited 85\%, whereas control EC/X\textsubscript{i}/V was inhibited 99\%. An antibody to factor V did not affect LPS-EC activity but did reduce prothrombinase activity by \approx 90\%. When isotype control monoclonal antibodies were tested in similar concentrations, no inhibition of thrombin generation was seen in either model. The effects of PMSF also differed, in that LPS-EC were more sensitive to this protease inhibitor than prothrombinase.

LPS-EC generated 6\% of thrombin generated by control EC and factors X\textsubscript{i}/V. Incubation of LPS-EC with activated protein C (10 \mu g/mL) reduced thrombin generation by 47\%. Incubation of LPS-EC or an extract of LPS-EC with a chromogenic substrate for factor X resulted in no substrate hydrolysis. These results suggest that the EC activator has serine protease characteristics (inhibition by PMSF), and may resemble factor X, in certain respects (inhibition by DEGR-chloromethyl ketone, a specific factor X inhibitor, inhibition by an antibody to factor X).

Figure 2 compares prothrombin cleavage by prothrombinase versus the LPS-inducible prothrombin activator. Aliquots of incubation mixtures were subjected to reduced SDS-PAGE and autoradiography. Control EC did not activate prothrombin in the absence of exogenous factors X\textsubscript{i}/V. How-
ever, LPS-treated EC, and control EC with factors X/V activated prothrombin in a similar manner.

Cycloheximide pretreatment of LPS-EC prevented inducible prothrombin activation. In this study, control EC generated <0.5 nmol/L thrombin, whereas LPS-EC generated 5 nmol/L thrombin. Preincubation of LPS-EC with cycloheximide (10 μg/mL) resulted in <0.5 nmol/L thrombin generated.

Factor Vα is a cofactor for factor Xα-catalyzed prothrombin activation. We investigated whether exogenous factor V would enhance activity of the inducible prothrombin activator. Figure 3 shows prothrombin concentration-response curves in which control EC incubated with factors X/V and prothrombin (Fig 3A) were compared with LPS-EC incubated with factor V and prothrombin (Fig 3B). The apparent Km of control EC and prothrombinase was 1.07 ± 0.06 μmol/L; the apparent Km of LPS-EC incubated with factor V and prothrombin was 1.28 ± 0.10 μmol/L.

To compare endothelial cell-dependent prothrombin activation in the control and LPS-EC models, experiments were performed with and without saturating levels of factors X/V and control EC (Table 2). Three concentrations of factors X/V were tested, 20 ng/mL X, 6 μg/mL V (100%), 10 ng/mL X, 3 μg/mL V (50%), and 2 ng/mL X, 0.6 μg/mL V (10%). As shown in Table 2, thrombin generation was directly related to concentrations of factors X/V. Comparison of thrombin generation by LPS-EC with exogenous factor V (468 nmol/L thrombin formed) with the data of Table 2 indicates that the inducible prothrombin activator (with exogenous factor V) generated 41% of thrombin generated by control EC and prothrombinase.

In the prothrombinase reaction, factor Vα is thought to be initially generated from factor Xα activation of factor Vα: factor Vα then mediates factor Xα-catalyzed prothrombin activation. We tested the hypothesis that LPS-EC could also activate factor V, to provide an activated cofactor for the inducible prothrombin activator. Exogenous factor V was incubated with control or LPS-EC, and factor V (Vα) activity measured in supernatants and cell lysates by clotting assay. Control supernatants possessed 388 ± 43 mU/mL factor V (Vα) activity, whereas LPS-EC supernatants showed 4,650 ± 300 mU/mL activity. Control cell lysates possessed 303 ± 31 mU/dish factor V (Vα) activity, whereas LPS-EC lysates showed 3,980 ± 103 mU/dish activity. Radioimmunoassay of EC factor V antigen levels showed control EC to have 69 ± 4 ng/dish (n = 4) and LPS-EC to have 71 ± 2 ng/dish (n = 4). These data suggest that LPS-EC can activate factor V.

Figure 4 shows a molecular weight estimation of the inducible prothrombin activator, as deduced from a gel-slice

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### Table 2. Thrombin Generation by Control EC and Varying Concentrations of Factors X/V

<table>
<thead>
<tr>
<th>Factor Xα (ng/mL)</th>
<th>Factor V (μg/mL)</th>
<th>Thrombin (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>20:6</td>
<td>1,142</td>
</tr>
<tr>
<td>50%</td>
<td>10:3</td>
<td>850</td>
</tr>
<tr>
<td>10%</td>
<td>2:06</td>
<td>352</td>
</tr>
<tr>
<td>0%</td>
<td>—</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Experiments were conducted as described in Materials and Methods using control EC, prothrombin (140 μg/mL) and factors X/V concentrations indicated in the table. Results shown are the mean of duplicate experiments. A factor Xα concentration of 20 ng/mL and factor V concentration of 8 μg/mL was arbitrarily defined as 100% (saturating levels).

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Fig 3. Comparison of thrombin generation by control EC and factors X/V versus LPS-treated EC and factor V. Cells were grown in 24-well trays, and concentrations of reactants were identical to that described in Fig 2, except that LPS-EC were also incubated with factor V (6 μg/mL). Aliquots were obtained and assayed for thrombin activity using a thrombin clotting time assay. (A) the Km of control EC with factors X/V was 1.07 ± 0.06 μmol/L; (B) the Km of LPS-EC with factor V was 1.28 ± 0.10 μmol/L.
Endothelial Cell Prothrombin Activator

The prothrombin activator has an apparent molecular weight of ~30 kDa. The inducible activator also has similarities and differences to factor Xα (Table 1). For example, dansyl-Glu-Gly-Arg chloromethyl ketone and an antibody to factor X inhibit both the inducible activator and prothrombinase. However, factor Xα has a molecular weight of 48 kDa, whereas the inducible activator has a molecular weight of ~30 kDa, and the prothrombin activator was more sensitive to PMSF inhibition than factor Xα. Additionally, the inducible activator is membrane-associated, whereas factor Xα is a plasma protease, and the activator did not hydrolyze a substrate specific for factor Xα. TAP also inhibited factor Xα-catalyzed prothrombin activation to a greater extent than the inducible activator.

The inducible EC activator also has properties similar to those of the murine monocyte enzyme.11 The monocyte enzyme cleaved prothrombin in a similar manner as factor Xα, was membrane associated, required induction by LPS or immune complexes, required calcium for maximal activity, and showed characteristics of a serine protease.11 The investigators of the murine monocyte enzyme did not identify enhancement of activity by factor V or report a molecular weight.11 Defining whether the inducible activator is factor Xα or a different protease awaits structural data on the purified protein. Our current data suggests that the inducible prothrombin activator has factor Xα-like activity.

Although an antibody to factor V did not inhibit prothrombin activation by LPS-EC, exogenous factor V enhanced activity of the inducible activator, and activated protein C-dependent activity of the activator. Human umbilical vein EC and bovine aortic EC do synthesize factor V, and it is possible that basal activity of the inducible activator is dependent on EC-synthesized factor V (Vα). In addition to activating prothrombin, LPS-treated EC also activated exogenous factor V 12-fold. Future studies using the purified prothrombin activator will permit us to show whether this protease is responsible for both prothrombin activation and factor V activation.

What are the implications of an inducible vascular prothrombin activator? Tissue factor-initiated factor X activation and subsequent factor Xα-catalyzed prothrombin activation would be downregulated by tissue factor pathway inhibitor.23 Although thrombin produced by the tissue factor mechanism might initiate intrinsic coagulation to generate additional thrombin,24 induction of a vascular cell prothrombin activator would also provide a source of thrombin activity associated with pathologic stimuli. It is notable that in a study by Levi et al25 in which primates were infused with endotoxin and parameters of activation of coagulation measured, the authors found paradoxical data regarding factor X activation in vivo (no factor IX or X activation peptides generated after endotoxin infusion), leading them to postulate that "other mechanisms coupled with tissue factor expression are required for endotoxin-induced prothrombin activation in vivo."25 The inducible prothrombin activator may constitute one of these mechanisms. Future studies will be directed toward purifying and identifying this procoagulant molecule.

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