Thrombin Receptor 14–Amino Acid Peptide Mediates Endothelial Hyperadhesivity and Neutrophil Adhesion by P-Selectin–Dependent Mechanism

Yasuo Sugama and Asrar B. Malik

Thrombin cleaves its receptor at arginine-41, resulting in the generation of a new receptor NH$_2$-terminus with the sequence SFLLRNPNKDYEPF. This peptide (TRP-14) may signal a variety of thrombin’s responses. We examined the effects of TRP-14 in inducing endothelial cell hyperadhesivity and neutrophil (PMN) adhesion to endothelial cell monolayers. Human umbilical vein endothelial cells (HUVECs) challenged with TRP-14 (10$^{-4}$ to 10$^{-5}$ M) produced concentration-dependent increases in endothelial adhesivity to PMN. In contrast, position 1 to 2 inverted peptide (FSLLRNPNKDYEPF) did not induce the response. The adhesion response was transient; that is, PMN adhesion increased within 15 minutes and decreased by 75 minutes after TRP-14 challenge of HUVECs. The transient endothelial adhesiveness paralleled the time course of P-selectin expression. TRP-14–induced release of P-selectin from intracellular stores may be a critical determinant of the response since treatment of endothelial cells with anti–P-selectin monoclonal antibody (mAb) G1 prevented the increase in PMN adhesion. Control nonneutralizing anti–P-selectin mAb S12 and mAb RR1/1 directed against intercellular adhesion molecule-1 (ICAM-1) on HUVECs were ineffective. The results indicate that the “tethered ligand” of the thrombin receptor created by the proteolytic action of thrombin on its receptor (i.e., TRP-14) signals increased endothelial adhesiveness by a P-selectin–dependent mechanism. Thrombin-induced PMN adhesion may involve formation of a new NH$_2$-terminus of the endothelial thrombin receptor with the sequence SFLLRNPNKDYEPF followed by activation of endothelial second messenger pathways and the transient expression of P-selectin. (Circulation Research 1992;71:1015–1019)

KEY WORDS • thrombin • neutrophils • human umbilical vein endothelial cells • endothelial adhesivity • P-selectin • thrombin receptor • tethered ligand

Thrombin mediates neutrophil adhesion to endothelial cells by promoting endothelial hyperadhesiveness.$^{1-3}$ Thrombin-induced intravascular neutrophil sequestration has been implicated as an important factor in tissue inflammatory responses such as the adult respiratory distress syndrome. The mechanism of thrombin’s action may involve binding of thrombin to a receptor on endothelial cell membrane and subsequent activation of second messenger pathways that in turn can induce the expression of endothelial adhesive molecules such as P-selectin.$^{6-8}$ P-Selectin, a constituent of the electron-dense Weibel-Palade bodies, is rapidly released in response to thrombin.$^{7,9}$ Studies have shown that the rapid component of the thrombin-induced increase in neutrophil adhesion (occurring within 15–30 minutes) is associated with P-selectin translocation to the plasma membrane and that the adhesion event can be inhibited by anti–P-selectin antibodies.$^{7,9}$

The recent cloning of a thrombin receptor indicates that thrombin binds to its receptor, which it cleaves after arginine 41 in the receptor’s NH$_2$-terminal portion, and thereby exposes a new NH$_2$-terminus that has been proposed to function as a “tethered ligand” for the receptor.$^{5}$ The binding of this “ligand” containing the terminal 14–amino acid peptide to an as yet undefined region of the thrombin receptor reproduces some of thrombin’s actions.$^{5}$ In the present study, we investigated the role of the 14–amino acid peptide (which can mimic thrombin’s effect such as platelet aggregation and fibroblast proliferation)$^{10}$ in inducing increased endothelial adhesivity to neutrophils.

Materials and Methods

Delbacco’s modified Eagle’s medium (DMEM), fetal bovine serum, and Hanks’ balanced salt solution (HBSS) were obtained from GIBCO, Grand Island, N.Y. The 14–amino acid thrombin receptor peptide (TRP-14) and a control peptide with positions 1 and 2 inverted were synthesized by Dr. Thomas T. Andersen, Peptide Synthesis Core, Albany Medical College, Al-
bany, N.Y. The anti-P-selectin monoclonal antibody (mAb) G1 and a control nonneutralizing anti-P-selectin mAb S12 were kindly provided by Dr. Rodger P. McEver, Department of Medicine, University of Oklahoma. An anti-intercellular adhesion molecule (ICAM-1) mAb RR1/1 was provided by Dr. Robert Rothlein, Boehringer-Ingelheim, Ridgefield, Conn. All mAbs were directed against the human antigens.

**Peptide Synthesis**

The 14–amino acid peptide SFLRNPNKDYEFP was synthesized as the C-terminal amide on a Biosearch 9500 peptide synthesizer using the solid phase method of Merrifield. The control peptide, in which the N-terminal position 1 and 2 amino acids were reversed (FSLRNPNKDYEFP), was also similarly synthesized. tert-Butyloxycarbonyl (tBOC)–derived amino acids (Advanced ChemTech, Louisville, Ky.) were used, and the side-chain protection was as follows: Ser (Bzl), Arg (Tos), Asp (OBzl), Lys (CIZ), Tyr (BrZ), and Glu (OBzl). The completed peptides were removed from the resin using the low-high method of Tam et al., washed with ethyl acetate, extracted into acetic acid, and lyophilized. The peptides were purified by reverse-phase chromatography on Sep-Pak Cartridges (Waters/Millipore, Bedford, Mass.). High-performance liquid chromatography and amino acid analysis indicated peptides of >98% purity and proper composition. The peptides were sequenced on a Protein 2090E enhanced gas phase sequencer, and the identified sequence was that expected for the synthetic peptides.

**Isolation of Neutrophils**

Human polymorphonuclear leukocytes (PMNs) were isolated from blood of normal volunteers that was supplied by the American Red Cross, Albany, N.Y. PMNs were isolated within 3 hours of obtaining the blood. The blood was sedimentated with the same volume of 4% dextran HBSS without Ca2+ and Mg2+ with 20 mM EDTA (disodium ethylenediamine tetraacetate) for 40 minutes at room temperature. After the sedimentation, the leukocyte-rich plasma layer was diluted in the same volume of HBSS with EDTA. The Ficoll-Paque (10 ml) (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) layered with diluted leukocyte-rich plasma was centrifuged for 30 minutes at room temperature at 1,350 rpm (400g). The pellet containing red blood cells and PMNs was resuspended, and after hypotonic lysis of red blood cells, the PMNs suspended in 1 ml/ml bovine serum albumin (BSA), 20 mM EDTA, 25 mM HEPES/HBSS were counted.

**Neutrophil Labeling**

The isolated PMNs were labeled with 51-sodium chromate (Cr) (New England Nuclear, Boston). The PMNs were incubated with 1 μCi of 51Cr per 10⁶ cells in a 5% CO₂ incubator at 37°C for 60 minutes, washed three times with HBSS, and resuspended on DMEM (2×10⁷ cells/ml).

**Endothelial Cultures**

Human umbilical vein endothelial cells (HUVECs) were cultured as described. HUVECs were seeded onto gelatin-coated 24-well plates (Corning, Corning, N.Y.) and grown to confluency.

**Adherence Assay**

HUVECs were incubated (37°C, 5% CO₂, and 98% humidity) for various times in DMEM without serum and with TRP-14. At the end of the incubation period, the endothelial monolayers were fixed with 1% paraformaldehyde/phosphate–buffered saline at room temperature for 15 minutes, and then washed three times with DMEM without serum. The 51Cr-labeled human PMNs (2×10⁶ cells/ml DMEM) were distributed at 1 ml per well over the confluent HUVECs and coincubated for 60 minutes at 37°C, 5% CO₂, and 98% humidity. The endothelial monolayers were then gently washed three times with DMEM without serum to remove nonadherent PMNs. The endothelial monolayers were kept overnight under 1 ml of 1N NaOH at 4°C. The cell lysates were scraped, collected in polypropylene test tubes, and counted for radioactivity in a Tm Analytical gamma counter.

**Anti–P-Selectin Monoclonal Antibody Binding Assay**

The time course of TRP-14–induced expression of GMP-140 on HUVECs was determined by the binding of 125I-labeled mAb G1 to HUVECs after TRP-14 challenge for varying durations. The anti–P-selectin mAb was iodinated using the chloramine-T method. HUVECs grown to confluency in 96-well plates were washed with DMEM without serum and incubated with either α-thrombin (10⁻⁴ M) (supplied by Dr. John Fenton, Albany Medical College, Albany, N.Y.) or TRP-14 (10⁻⁴ M) for 15, 30, 45, 60, 75, 90, or 120 minutes. At the end of the incubation periods, the cells were fixed with 1% paraformaldehyde/phosphate–buffered saline at room temperature for 15 minutes. The HUVECs were then washed in DMEM containing 10% serum, 125I-labeled mAb G1 (10 μg/ml DMEM with DMEM containing 10% serum (75 μl per well), and the cells were allowed to incubate for 60 minutes at 4°C. The specific activity of 125I-labeled G1 was 3.75 μCi/μg. The cells were gently washed three times with DMEM with serum to remove nonbinding 125I-G1. The cells were kept overnight in 150 μl of 1N NaOH at 4°C, after which the cell lysate was scraped, collected in test tubes, and counted for radioactivity in a Tm Analytical gamma counter. The specific binding of G1 was determined by adding 30-fold excess unlabeled G1. The 125I-G1 binding data were normalized to cell protein measured in control cells and after either thrombin or TRP-14 challenge.

**Data Analysis**

Statistical analysis was performed with the Student’s t test, and p<0.05 was considered significant. The results are given as mean±SEM.

**Results**

Figure 1 shows the effects of TRP-14 (the 14–amino acid peptide that comprises the new NH₂-terminus or the ligand portion of the thrombin receptor) on the expression of endothelial adhesiveness. The endothelial cells were treated with varying concentrations of TRP-14 and then fixed with 1% paraformaldehyde before applying 51Cr-labeled neutrophils. The results indicate an increase in endothelial adhesiveness occurring between TRP-14 concentrations of 10⁻⁵ M and...
The time course of the increase in endothelial adhesiveness is shown in Figure 2. Endothelial cells were treated with TRP-14 for the indicated periods and fixed with 1% paraformaldehyde before applying neutrophils. Endothelial adhesiveness increased within 15 minutes after TRP-14 challenge and decreased to basal levels after treatment of endothelial cells for periods longer than 75 minutes (Figure 2), indicating the reversibility of the response. Figure 2 also shows the effects of treatment of endothelial cells with the mAb G1 directed against human P-selectin. MAb G1 inhibited neutrophil adhesion to the TRP-14-challenged endothelial cells (Figure 2). In contrast, the anti-P-selectin control mAB S12 and the anti–ICAM-1 mAb RR1/1 were ineffective in preventing neutrophil adhesion to TRP-14–treated endothelial cells (Table 2).

Figure 3 shows the specific binding of 125I-labeled anti–P-selectin mAb G1 after challenge with α-thrombin (a positive control) or TRP-14. An increase in specific binding of 125I-G1 was evident within 15 minutes after either α-thrombin or TRP-14 challenge, indicating rapid P-selectin expression. In both cases, the binding decreased to control levels within 75 minutes after TRP-14 challenge.

Table 2. Effects of Anti–GMP-140 Monoclonal Antibody G1, Control Anti–P-Selectin Monoclonal Antibody S12, and Anti–ICAM-1 Monoclonal Antibody RR 1/1 on Polymorphonuclear Leukocyte Adhesion to Human Umbilical Vein Endothelial Cells Treated for 15 Minutes With Either TRP-14 or α-Thrombin

<table>
<thead>
<tr>
<th>PMN adhesion (%)</th>
<th>TRP-14 (5×10⁻⁵ M)</th>
<th>α-Thrombin (10⁻⁸ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.9±3.3</td>
<td>21.3±3.2</td>
</tr>
<tr>
<td>mAb G1 (10 µg/ml)</td>
<td>3.3±0.5*</td>
<td>2.0±0.5*</td>
</tr>
<tr>
<td>mAb S12 (10 µg/ml)</td>
<td>22.7±1.1</td>
<td>18.2±1.1</td>
</tr>
<tr>
<td>mAb RR1/1 (10 µg/ml)</td>
<td>21.5±2.7</td>
<td>. . .</td>
</tr>
</tbody>
</table>

Values are shown as mean±SEM; n=8 for each condition. Human umbilical vein endothelial cells (HUVECs) were fixed with 1% paraformaldehyde, and then treated with these antibodies 15 minutes before applying polymorphonuclear leukocytes (PMNs). Basal PMN adhesion to fixed non-activated HUVECs was 2.3±0.3%. Similar results were obtained in three experiments. TRP-14, thrombin receptor peptide; mAb, monoclonal antibody. *p<0.05 from control.

Table 1. Comparison of Neutrophil Adhesion Induced With TRP-14 and a Control Peptide in Which the N-terminal Amino Acids Are Reversed

<table>
<thead>
<tr>
<th>Peptides</th>
<th>PMN adhesion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>SFLLRNPDKYEPF (5×10⁻⁵ M)</td>
<td>29.1±2.6*</td>
</tr>
<tr>
<td>SFLLRNPDKYEPF (10⁻⁴ M)</td>
<td>1.5±0.2</td>
</tr>
</tbody>
</table>

Values are shown as mean±SEM; n=8 for each group. Human umbilical vein endothelial cells were fixed with paraformaldehyde before applying neutrophils (see “Materials and Methods”). Similar results were obtained in three experiments. *Different from basal adhesion.
by guest on October 27, 2017 http://circres.ahajournals.org/ Downloaded from

FIGURE 3. Line graph shows binding of 125I-labeled anti-P-selectin monoclonal antibody G1 to human umbilical vein endothelial cells. Cells were treated with either α-thrombin (10–4 M) (positive control) or thrombin receptor peptide (TRP-14) (10–4 M) for the indicated periods (see "Materials and Methods"). Values are shown as mean ± SEM; n = 4 for each condition.

Discussion

We have previously shown that thrombin is a potent mediator of neutrophil sequestration in the microcirculation.4 Thrombin may mediate its effects by two likely mechanisms. Thrombin causes deposition of fibrin in microvessels, which provides a substrate for neutrophil attachment to the intravascular fibrin.19 The "entrapment" of neutrophils in the fibrin meshwork may be mediated by binding of the neutrophil CD18 integrin to its "counterreceptor" on fibrin.19 The second mechanism involves expression of endothelial adhesiveness secondary to the upregulation of endothelial cell surface molecules, P-selectin2–7 and ICAM-1.3 Although studies have shown that thrombin can upregulate these adhesion molecules,1,9 the mechanisms of the response remain unclear.

In the present study, we examined the role of the 14-amino acid thrombin receptor peptide (TRP-14), which contains the sequence of the NH2-terminus of the thrombin receptor created by proteolytic cleavage by thrombin of its receptor after arginine-41.5 TRP-14 has some agonist properties similar to thrombin such as its ability to induce platelet aggregation,5 fibroblast proliferation,10,20 and prostacyclin generation from endothelial cells.21 The present results indicate that this peptide is also capable of inducing an increase in endothelial adhesiveness to neutrophils secondary to the expression of P-selectin. The control 14-amino acid peptide in which the NH2-terminal amino acids in positions 1 and 2 were reversed (FSLLRNPDKYEPF) did not cause neutrophil adhesion.

The time course of P-selectin expression after challenge of endothelial cells with TRP-14 or α-thrombin was similar in that both caused an increase in the specific binding of the anti-P-selectin mAb G1 within 15 minutes of challenge, and the P-selectin expression was downregulated within 75 minutes. The latter process may reflect internalization or "shedding" of the P-selectin antigen.7,9 Since the endothelial cells were fixed with paraformaldehyde before assessment of P-selectin expression and neutrophil adhesion, the TRP-14–induced increase in endothelial adhesiveness cannot be explained by secondary mediators released by endothelial cells.

The neutrophil adhesion response to TRP-14 occurred rapidly (the maximum response was evident within 15 minutes) and was short-lived (the response decayed by 75 minutes). The time course of the response paralleled the expression of P-selectin on the surface of endothelial cells. Incubation with TRP-14 for periods longer than 75 minutes significantly diminished neutrophil adhesion from its peak value consistent with downregulation of P-selectin. Thrombin is known to rapidly induce the release of P-selectin from Weibel-Palade bodies in endothelial cells resulting in translocation of this adhesive molecule to the plasma membrane where it promotes neutrophil adhesion.6,7,22 Since P-selectin expression occurred within the same time course as thrombin’s response and treatment of the paraformaldehyde-fixed endothelial cells with mAb G1 prevented the TRP-14–induced neutrophil adhesion, TRP-14 and thrombin may activate P-selectin release in the same manner. The second messengers responsible for P-selectin upregulation are not known. Increase in cytosolic Ca2+ signaled by TRP-14 binding to endothelial cells may be a critical event.23 In the present study, the increase in endothelial adhesiveness could not be ascribed to ICAM-1 since an anti–ICAM-1 mAb RR1/1 did not alter TRP-14–induced neutrophil adhesion.

The transient neutrophil adhesion response to TRP-14 observed in the present study is different from longer-lasting increase in endothelial adhesiveness observed with thrombin challenge.1,2 This suggests that thrombin acts by mechanisms, in addition to thrombin’s proteolytic activity on this thrombin receptor. There may be other as yet undefined thrombin receptors or the proteolytic activity of thrombin may activate additional endothelial adhesion molecules such as ICAM-1,3 which can sustain the endothelial adhesion response to thrombin.

In conclusion, we have shown that TRP-14 (which contains the amino acid sequence of NH2-terminus of the thrombin receptor after its cleavage at arginine-41) increases endothelial adhesiveness and mediates transient neutrophil adhesion to endothelial cells by a P-selectin–dependent mechanism. The increase in endothelial adhesiveness is associated with upregulation of P-selectin on the endothelial cell surface. The results suggest that receptor antagonist peptides directed against TRP-14 binding sites on the endothelial cell membrane may be potentially useful anti-inflammatory agents in preventing neutrophil adhesion and sequestration in the microcirculation.

References


![Graph showing binding of 125I-labeled anti-P-selectin monoclonal antibody G1 to human umbilical vein endothelial cells.](image-url)
Y Sugama and A B Malik

Thrombin receptor 14-amino acid peptide mediates endothelial hyperadhesivity and neutrophil adhesion by P-selectin-dependent mechanism.

Circ Res. 1992;71:1015-1019
doi: 10.1161/01.RES.71.4.1015

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/71/4/1015

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/