Aprotinin inhibits proinflammatory activation of endothelial cells by thrombin through the protease-activated receptor 1

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Objective: Thrombin is generated in significant quantities during cardiopulmonary bypass and mediates adverse events, such as platelet aggregation and proinflammatory responses, through activation of the high-affinity thrombin receptor protease-activated receptor 1, which is expressed on platelets and endothelium. Thus antagonism of protease-activated receptor 1 might have broad therapeutic significance. Aprotinin, used clinically to reduce transfusion requirements and the inflammatory response to bypass, has been shown to inhibit protease-activated receptor 1 on platelets in vitro and in vivo. Here we have examined whether aprotinin inhibits endothelial protease-activated receptor 1 activation and resulting proinflammatory responses induced by thrombin.

Methods: Protease-activated receptor 1 expression and function were examined in cultured human umbilical vein endothelial cells after treatment with α-thrombin at 0.02 to 0.15 U/mL in the presence or absence of aprotinin (200-1600 kallikrein inhibitory units/mL). Protease-activated receptor 1 activation was assessed by using an antibody, SPAN-12, which detects only the unactivated receptor, and thrombin-mediated calcium fluxes. Other thrombin-dependent inflammatory pathways investigated were phosphorylation of the p42/44 mitogen-activated protein kinase, up-regulation of the early growth response 1 transcription factor, and production of the proinflammatory cytokine interleukin 6.

Results: Pretreatment of cultured endothelial cells with aprotinin significantly spared protease-activated receptor 1 receptor cleavage ($P < .0001$) and abrogated calcium fluxes caused by thrombin. Aprotinin inhibited intracellular signaling through p42/44 mitogen-activated protein kinase ($P < .05$) and early growth response 1 transcription factor ($P < .05$), as well as interleukin 6 secretion caused by thrombin ($P < .005$).

Conclusions: This study demonstrates that endothelial cell activation by thrombin and downstream inflammatory responses can be inhibited by aprotinin in vitro through blockade of protease-activated receptor 1. Our results provide a new molecular basis to help explain the anti-inflammatory properties of aprotinin reported clinically.

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Cardiopulmonary bypass (CPB), although remaining an indispensable asset in cardiac surgery, is associated with significant thrombin generation in the bypass circuit.[1,2] Sternotomy might also contribute to the increase in thrombin.[3] Thrombin is a multifunctional serine protease that plays a crucial role in the

CSP

Abbreviations and Acronyms

CPB = cardiopulmonary bypass
Egr = early growth response
HRP = horseradish peroxidase
HUVEC = human umbilical vein endothelial cell
IL = interleukin
KIU = kallikrein inhibitory units
MAP = mitogen-activated protein
PAR = protease-activated receptor
SD = standard deviation
TRAP = thrombin receptor agonist peptide

regulation of hemostasis and provides a link between the coagulation and inflammatory systems. Its presence during bypass leads to platelet dysfunction and excessivet bleeding postoperatively and might be implicated in the systemic inflammatory response and perioperative stroke.[3-5]

Thrombin can trigger cellular responses in a variety of cell types through activation of a family of 7 transmembrane-spanning G protein–coupled receptors known as protease-activated receptors (PARs). Thrombin activates PAR1 by means of proteolytic cleavage, exposing a new amino terminus that serves as a tethered ligand by binding to sites within the body of the receptor.[6] Synthetic peptides corresponding to the amino terminus of the tethered ligand can act as ligand mimetics and bypass the need for receptor cleavage. The best characterized example of such a peptide is the thrombin receptor agonist peptide (TRAP) 6, which is specific for human PAR1.[7]

Endothelial cells play a critical role in the regulation of thrombosis and inflammation, which can become dysregulated after CPB.[4] Thrombin signaling in the endothelium regulates permeability, vascular tone, leukocyte trafficking and inflammation, formation of new vessels, and hemostasis. Of the 3 human thrombin receptors, PAR1, PAR3, and PAR4, identified thus far, PAR1 is the predominant form expressed on endothelial cells[8] and is also widely expressed on platelets, on smooth muscle cells, and in the central nervous system.[9-11] Activation of PAR1 by thrombin has been shown to mediate many adverse events seen after CPB, such as platelet aggregation, inflammatory cytokine secretion, and neurodegeneration.[10-13] PAR1 activation triggers a number of signaling pathways that include G protein–coupled calcium flux and phosphorylation (ie, activation) of the signaling protein p42/44 mitogen-activated protein (MAP) kinase, protein kinase C, phosphatidylinositol 3 kinase, and the early growth response (Egr) 1 transcription factor.[10,13,14] There is presently great interest in blocking the signaling pathways downstream of PAR1 pharmacologically, and several classes of PAR1 antagonists are in preclinical trials.[15,16]

Interleukin (IL) 6 is an acute-phase inflammatory cytokine that plays a critical role in leukocytosis, thrombosis, inflammation, and neurodegeneration.[17] It is secreted by activated endothelial cells, macrophages, fibroblasts, and adipocytes. Plasma IL-6 levels are strongly induced at 3 to 4 hours after CPB, and IL-6 was shown to be the most highly upregulated gene (>41-fold increase) by means of microarray analysis of 12,625 genes in patients undergoing CPB and cardioplegic arrest.[18,19] A promoter polymorphism that leads to abnormally high levels of IL-6 has been linked to an exacerbated systemic inflammatory response and atrial fibrillation after CPB.[20] Because thrombin is known to stimulate IL-6 secretion by endothelial cells in vitro through PAR1,[21] this source of IL-6 production might be of clinical relevance to the inflammatory complications of CPB.

The protease inhibitor aprotinin, already approved for clinical use to limit transfusion requirements in cardiac surgery, has been shown to block PAR activation on platelets, both in vitro and in vivo.[22-24] We hypothesize that the anti-inflammatory properties of aprotinin in CPB might be mediated, at least in part, through an ablation of thrombin-mediated PAR1 signals on endothelial cells. In the present study we demonstrate that aprotinin blocks PAR1-mediated signaling and IL-6 secretion in endothelial cells in vitro.

Materials and Methods

Antibodies

α-Thrombin and anti-β-tubulin antibody were purchased from Sigma-Aldrich (Poole, United Kingdom). Polyclonal rabbit antibodies against phosphorylated or total p42/44 MAP kinase protein and secondary horseradish peroxidase (HRP)–linked sheep anti-rabbit antibody were purchased from Cell Signaling Technologies (Hitchin, United Kingdom). Anti-Egr-1 antibody was purchased from Santa Cruz Biotechnology, Inc (Middlesex, United Kingdom). Secondary HRP-linked sheep anti-mouse antibody was purchased from Amersham Pharmacia Biosciences (Chalfont St Giles, United Kingdom). TRAP-6 was purchased from Bachem (UK) Ltd (St Helens, United Kingdom).

Endothelial Cells

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins by means of collagenase II digestion (0.1%; Boehringer-Mannhein, Lewes, Sussex, United Kingdom), as previously described.[13] HUVECs were cultured in 75-cm^2 flasks and used between passages 2 and 4. At confluence, cells were detached with cell dissociation medium (Sigma Aldrich), a trypsin-free detachment step that avoids possible PAR activation by trypsin.

PAR1 Receptor Cleavage Assay

Receptor cleavage after thrombin activation was assessed by means of flow cytometry with an antibody, SPAN-12 (Beckman-Coulter, Luton, United Kingdom), that detects only the intact (ie, unactivated) PAR1 receptor. Loss of SPAN-12 staining therefore provides a linear measure of receptor activation. SPAN-12 staining was carried out on freshly passaged cells in suspension after α-thrombin stimulation (0.02 U/mL) for 5 minutes at 37°C with or without 200 kallikrein inhibitory units (KIU)/mL aprotinin. All

steps after addition of SPAN-12 antibody were carried out on ice to prevent internalization of cleaved receptor. SPAN-12 staining (10 μg/mL) was performed for 15 minutes, followed by 3 washes in ice-cold phosphate-buffered saline. Secondary antibody staining with fluorescein isothiocyanate–conjugated goat anti-mouse IgG antibody (1:64 dilution) was carried out for 15 minutes, followed by 3 washes in ice-cold phosphate-buffered saline and fixation with 2% paraformaldehyde. Flow cytometric analysis was performed with an EPICS XL flow cytometer (Coulter Electronics Ltd, Luton, United Kingdom). SPAN-12 staining was expressed in units of relative fluorescent intensity (RFI), which are calculated by dividing the mean fluorescent staining intensity obtained with SPAN-12 antibody by the staining intensity of a class-matched control antibody (ie, an RFI of 1.00 is equivalent to no detectable expression). Results were presented as mean SPAN-12 expression (RFI units) ± standard deviation (SD) from 9 experiments.

Calcium Flux

Endothelial cells in suspension were loaded with Fura-2 AM (5 μmol/L; Molecular Probes Inc, Eugene, Ore) for 1 hour at 37°C and adjusted to 4×10^6 cells/mL. Intracellular Ca^{2+} fluxes were determined by measuring Fura-2 fluorescence at 340 nm excitation and 510 nm emission with an LS 50 B spectrofluorimeter (Perkin Elmer, Beaconsfield, United Kingdom).

Immunoblotting

Western blotting was carried out as previously described.[25] Briefly, HUVECs were serum starved for 3 hours (in medium M199 supplemented with 2% bovine serum albumin) before stimulation with thrombin (0.15 U/mL). Preliminary experiments indicated that maximum p42/44 protein phosphorylation occurred at 10 minutes and Egr-1 upregulation occurred at 1 hour after thrombin addition, and cell lysates were therefore collected at 10 minutes in the case of p42/44 MAP kinase and 1 hour in the case of Egr-1 in buffer containing 50 mmol/L Tris/HCL (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 25 mmol/L sodium deoxycholic acid, 4 mmol/L ethylenediamine tetraacetic acid, 200 μmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, and 5% protease inhibitor cocktail (Sigma Aldrich). The protein content in lysates was first determined by using a Bio-Rad D_c protein assay (Bio-Rad, Hercules, Calif) to achieve equal protein loading into wells. Proteins were resolved by means of sodium dodecylsulfate–polyacrylamide gel electrophoresis and semidry blotted onto Immobilon-P membranes (Millipore Corp, Bedford, Mass). The membrane was then incubated with relevant primary antibodies overnight at 4°C, washed 3 times in Tris-buffered saline/0.1% Tween 20, and incubated for 1 hour at room temperature with an appropriate HRP-labeled secondary antibody. Blots were developed with enhanced chemiluminescence reagent (Amersham Biosciences) and exposed to autoradiography film. Films were scanned densitometrically with a ChemiImager 500 (Alpha Innotech Corp, San Leandro, Calif) to obtain a signal intensity in units of integrated density value. The specific signal (ie, phosphorylated p42/44 MAP kinase or Egr-1) was then corrected for any unequal protein content between lanes by stripping the membrane and reprobing for a constitutive control protein (ie, total p42/44 or β-tubulin, respectively). The integrated density value ratio of phosphorylated p42/44 to total p42/44 or Egr-1 to β-tubulin was calculated and expressed as the mean ± SD from 3 experiments.

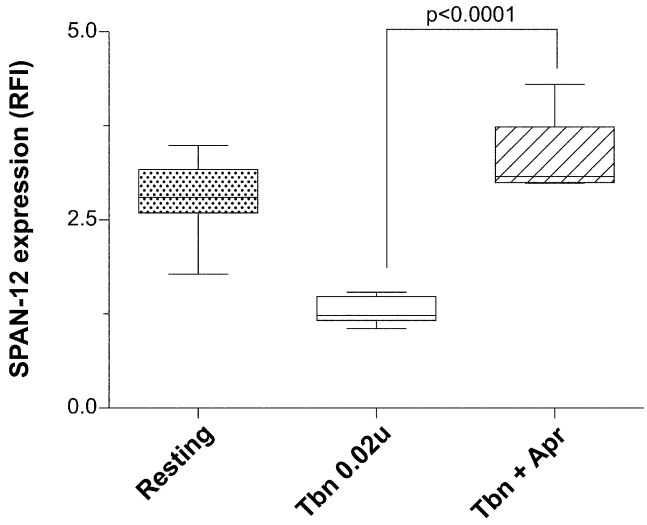

Figure 1. Effect of aprotinin on protease-activated receptor 1 *(PAR1)* receptor cleavage. Human umbilical vein endothelial cells were treated in suspension with α-thrombin (*Tbn*; 0.02 U/mL) for 10 minutes at 37°C with or without aprotinin *(Apr)* at 200 KIU/mL. Cleavage of PAR1 was monitored by means of flow cytometry with an antibody, SPAN-12, that detects only the intact (ie, unactivated) receptor. Results are expressed in units of relative fluorescent intensity *(RFI)*, which are calculated by dividing the mean fluorescent staining intensity obtained with SPAN-12 antibody by the staining intensity obtained with a class-matched (IgG_1) control antibody. *Boxes* show medians and quartiles, and *whiskers* indicate 5th and 95th percentiles (n = 9 experiments).

IL-6 Enzyme-linked Immunosorbent Assay

HUVECs were treated for 4 hours with 0.15 U/mL thrombin in the presence or absence of 1600 KIU/mL aprotinin. Supernatants were collected at 4 hours, centrifuged to remove cell debris, and assayed for IL-6 levels by using the enzyme-linked immunosorbent assay technique (Duosets; R&D Systems, Abingdon, United Kingdom), according to the manufacturer's recommendations. Results are expressed as the mean IL-6 concentration (in picograms per milliliter) ± SD from 7 experiments.

Statistics

Statistical comparisons between thrombin- and aprotinin-treated conditions were analyzed by using a paired Student *t* test.

Results

PAR1 Receptor Cleavage

The 7-transmembrane thrombin receptor PAR1 is activated by proteolytic cleavage of its exodomain, causing G protein–coupled signaling into the cell.[6] PAR1 activation was monitored by detecting receptor cleavage with an antibody,

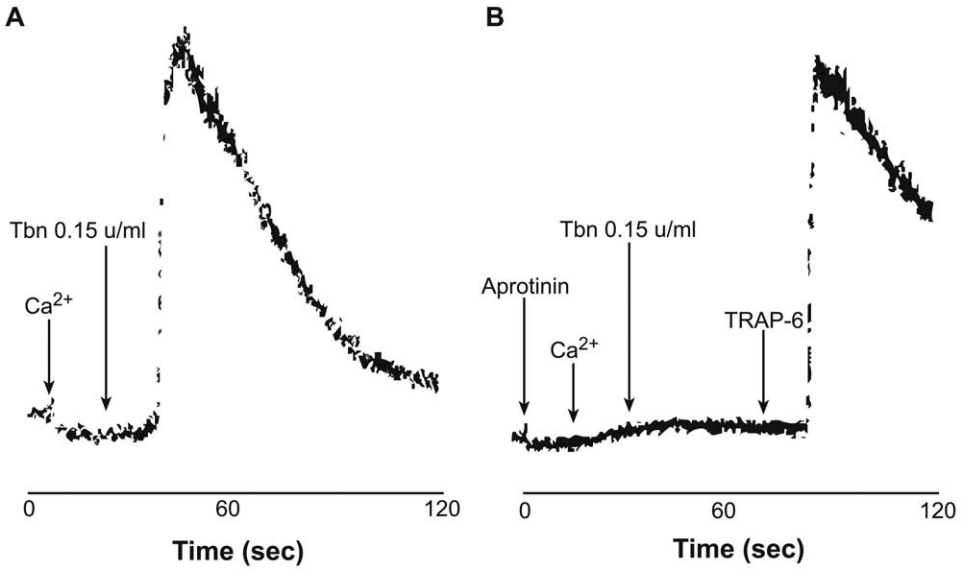

Figure 2. Effect of aprotinin *(Apr)* on thrombin *(Tbn)*–induced calcium fluxes. Human umbilical vein endothelial cells in suspension were loaded with the calcium fluorophore Fura-2 AM and monitored for intracellular calcium fluxes spectrophotometrically after addition of thrombin (0.15 U/mL; A) or thrombin plus aprotinin (1600 KIU/mL; B) and thrombin receptor–activating peptide 6 *(TRAP-6)*. Traces shown are representative of 3 similar experiments.

SPAN-12, that recognizes only the intact and unactivated receptor. Proteolytic activation of PAR1 took place at low concentrations of thrombin (0.02 U/mL) in cultured endothelial cells and was abrogated by treatment with aprotinin at 200 KIU/mL (Figure 1).

Calcium Flux

Thrombin induction of intracellular Ca^{2+} flux was measured as an indicator of PAR1 signal transduction.[10,22] Ca^{2+} fluxes were monitored spectrofluorimetrically in Fura-2–loaded cells, revealing a sharp calcium spike at 20 seconds after stimulation with thrombin used at 0.15 U/mL (Figure 2, *A*). This was completely abrogated by pretreatment with 1600 KIU/mL aprotinin (Figure 2, *B*). PAR1 receptors remained competent for signaling, however, because bypassing the proteolytic cleavage step with the synthetic activating peptide TRAP-6 induced a robust Ca^{2+} flux. Therefore inhibition of PAR1 cleavage by aprotinin abrogated thrombin-induced Ca^{2+} signals in endothelial cells.

p42/44 MAP Kinase Signaling Protein Activation

One of the key signaling pathways activated by thrombin is the MAP kinase pathway, which includes the p42/44 family.[13,14] To investigate whether aprotinin inhibits this pathway, phosphorylation of p42/44 was studied as a measure of activation. Activation of the p42/44 kinase, as detected by means of Western immunoblotting with a phospho-specific anti-p42/44 antibody, was induced by incubation of HUVECs with thrombin (0.15 U/mL) for 10 minutes. Aprotinin (1600 KIU/mL) suppressed thrombin-dependent phosphorylation of p42/44 (Figure 3, *A*). A ratio of phosphorylated versus total p42/44 protein was determined from densitometric scanning of blots to quantitate activation of p42/44 by thrombin and to confirm the inhibitory effect of aprotinin (Figure 3, *B*).

Egr-1 Transcription Factor Upregulation

Activation of p42/44 MAP kinase by thrombin is known to increase expression of the transcription factor Egr-1.[14] We therefore investigated Egr-1 protein levels in HUVECs by means of Western blot analysis, with β-tubulin as a loading control. This revealed upregulation of Egr-1 at 1 hour after thrombin addition (0.15 U/mL); however, this effect was abrogated in the presence of 1600 KIU/mL aprotinin (Figure 4).

IL-6 Secretion

Having demonstrated that aprotinin can inhibit thrombin-dependent signaling in endothelial cells, we set out to determine whether this inhibition could have functional relevance. An important target downstream of Egr-1 is the proinflammatory cytokine IL-6, which is strongly induced clinically after CPB.[18,19] IL-6 levels were therefore determined in HUVEC culture supernatants. This demonstrated

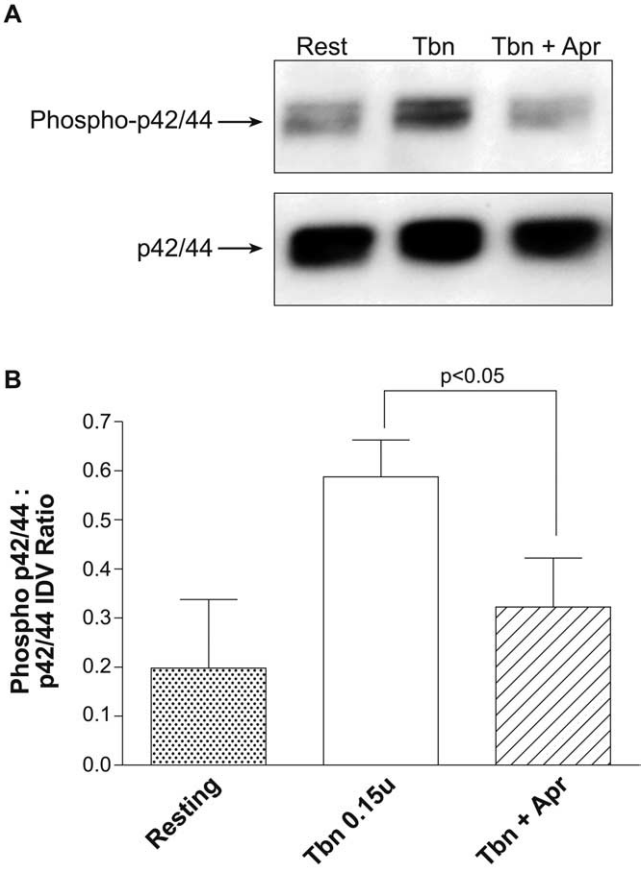

Figure 3. Effect of aprotinin *(Apr)* on thrombin *(Tbn)*–induced phosphorylation of p42/44 mitogen-activated protein kinase signaling protein. A, Endothelial cells were stimulated with thrombin (0.15 U/mL) or thrombin plus 1600 KIU/mL aprotinin for 10 minutes before lysis and immunoblotting for phosphorylated (ie, activated) p42/44 mitogen-activated protein kinase signaling protein. After detection with a horseradish peroxidase–conjugated secondary antibody and chemiluminescence, blots were stripped and reprobed for total p42/44 protein. B, The intensity of each band, in units of integrated density value *(IDV)*, was determined from densitometric scanning of blots, and the ratio of phosphorylated versus total p42/44 protein is shown as the mean IDV ratio ± standard deviation from 3 experiments.

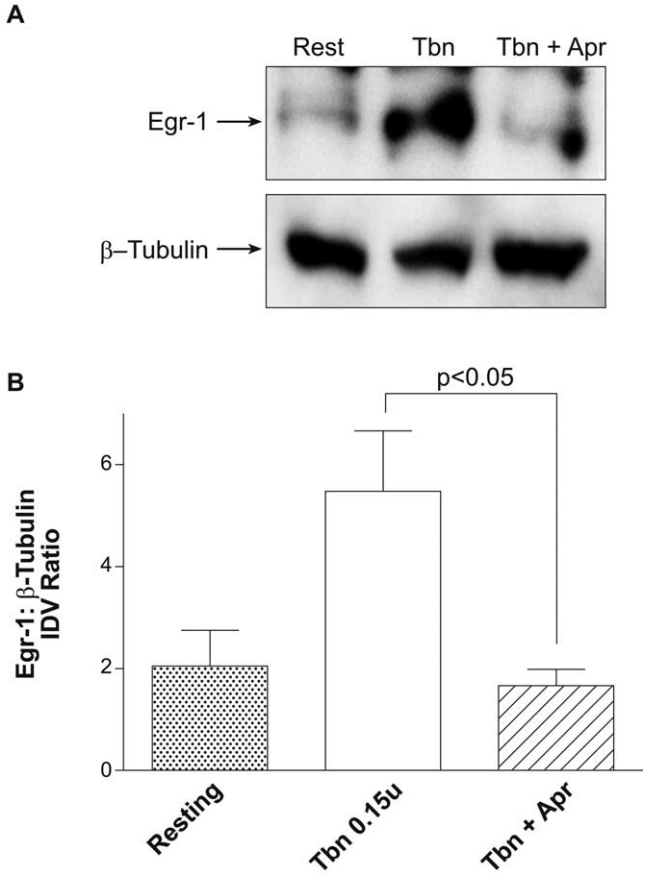

Figure 4. Effect of aprotinin *(Apr)* on thrombin *(Tbn)*–induced early growth response 1 *(Egr-1)* transcription factor upregulation. A, Endothelial cells were stimulated with thrombin (0.15 U/mL) or thrombin plus 1600 KIU/mL aprotinin for 1 hour before lysis and immunoblotting for Egr-1 protein. β-Tubulin is shown as a loading control. B, The ratio of Egr-1/β-tubulin protein was measured by means of densitometric scanning of blots and is shown as the mean integrated density value ratio *(IDV)* ± standard deviation from 3 experiments.

that thrombin (0.15 U/mL) induced IL-6 secretion at 4 hours; however, aprotinin (1600 KIU/mL) significantly inhibited this secretion (Figure 5).

Discussion

The anti-inflammatory properties of aprotinin in CPB have been recognized for more than 10 years[26-29]; however, the molecular mechanisms through which this is achieved have remained elusive. Inhibition of contact activation of platelets and neutrophils, indirect effects through kallikrein inhibition, and blockade of leukocyte extravasation have all been proposed on the basis of experimental models of inflammation.[26,28-32] Clinically, aprotinin also diminishes neutrophil accumulation and IL-8 secretion in the lung after CPB.[33] However, specific receptor mechanisms have only been recognized on platelets through targeting of the PAR1 receptor in vitro and in vivo.[22-24] Because the same receptor is present on endothelial cells and mediates proinflammatory signaling pathways due to thrombin, the present study examined whether aprotinin targeted endothelial PAR1 in vitro.

We have demonstrated that a cascade of thrombin-induced signaling events in cultured endothelial cells, involving PAR1 receptor cleavage, Ca^{2+} flux, p42/44 phosphorylation, Egr-1

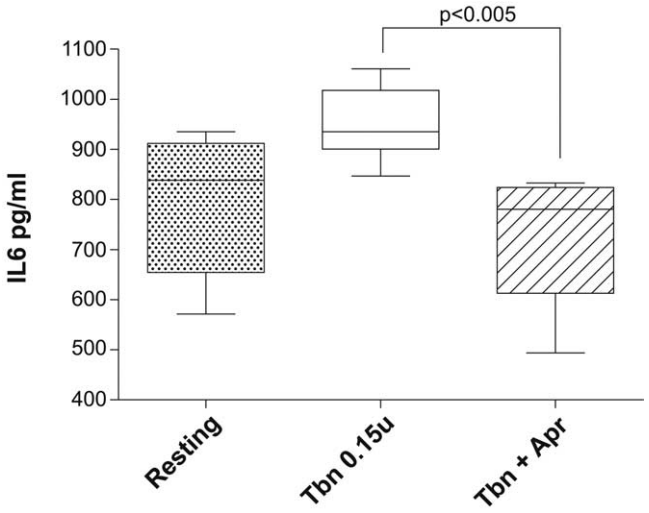

Figure 5. Effect of aprotinin *(Apr)* on thrombin *(Tbn)*–induced interleukin 6 *(IL-6)* secretion. Endothelial cell monolayers were established in 25-cm^2 flasks and stimulated for 4 hours in the presence of thrombin (0.15 U/mL) with or without 1600 KIU/mL aprotinin. Supernatants were collected, and levels of secreted IL-6 were determined by means of enzyme-linked immunosorbent assay. *Boxes* show medians and quartiles, and *whiskers* indicate 5th and 95th percentiles (n = 7 experiments).

upregulation, and IL-6 secretion, is blocked by aprotinin. The initial receptor cleavage event was blocked at 200 KIU/mL aprotinin, which is equivalent to the full dose regimen used clinically in CPB. Higher concentrations of aprotinin (1600 KIU/mL) were needed to block other downstream signaling events, but this might have been an artifact of the in vitro model system because 8-fold higher concentrations of thrombin were needed for downstream signaling in endothelial cells. The need for higher thrombin levels to fully activate signaling pathways might be due to a higher signaling threshold in cultured HUVECs, as previously recognized.[14] This represents a limitation of the present study, and it will be important to validate the molecular mechanisms identified here in vivo at clinically relevant doses of aprotinin.

Experiments with the anti-PAR1 antibody SPAN-12 and calcium flux show that aprotinin blocks the receptor cleavage step in PAR1 activation. Inhibition of calcium flux was restored in the presence of the agonist polypeptide TRAP-6. Because TRAP-6 bypasses the need for receptor cleavage during activation, this demonstrates unequivocally that aprotinin targets the proteolytic cleavage step in PAR1 activation. This is the same mechanism of action as described in platelets[22,23]; however, it is distinct from the peptidomimetic class of PAR1 antagonists, which do not affect receptor cleavage but block signal transduction.[15,16] As stated previously,[23] we do not believe that aprotinin directly inhibits the serine protease catalytic activity of thrombin in solution because it has a low inhibitory constant for thrombin,[34] and our working hypothesis is that aprotinin blocks the rate-limiting step of thrombin binding to the PAR1 receptor.

The anti-inflammatory potency of aprotinin on endothelial cells is underlined by its inhibition of IL-6 secretion, a potent proinflammatory cytokine upregulated during bypass.[18,19] Interestingly, there might also be a link between IL-6 and neurodegeneration and stroke. A promoter polymorphism in IL-6 is overrepresented in patients with a history of ischemic stroke, and high plasma IL-6 levels are associated with neurologic worsening immediately after stroke.[35,36] Although the source of IL-6 after bypass is unclear, therapeutic blockade of endothelial PAR1 fits well as a molecular mechanism to explain, at least in part, the anti-inflammatory and antistroke properties reported for aprotinin clinically.[26,27,29,33,37]

In conclusion, our results provide the first evidence that endothelial PAR1 can be targeted by aprotinin, as on platelets. Although further studies will be required to validate this mechanism of action in vivo, it is likely that endothelial PAR1 blockade might explain many of the anti-inflammatory properties of this drug reported clinically.

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