

Thrombin induces endothelial cell-surface exposure of the plasminogen receptor annexin 2

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Summary

Cell-surface annexin 2 (A2) and its ligand p11 have been implicated in fibrinolysis because of their ability to accelerate tissue plasminogen activator (tPA)-mediated activation of plasminogen to plasmin. Because thrombin is a potent cell modulator obligately produced at the site of clot formation, we hypothesized that the amount of cell-surface A2 and p11 might be altered by thrombin with consequent effects on plasmin generation. In support of this hypothesis, immunofluorescence microscopy and hydrophilic biotinylation experiments showed that both A2 and p11 were significantly increased on the surface of human umbilical vein endothelial cells (HUVECs) treated with thrombin (0.8–8 nM) for 5 minutes followed by 1 hour at 37°C. Intracellular immunofluorescence microscopy and immunoblot analyses of whole cell extracts revealed increased p11 but unchanged A2 in response to thrombin, suggesting that transbilayer trafficking of A2 might be

controlled by p11. The thrombin receptor-activating peptide (TRAP) similarly affected cells, demonstrating that cell signaling at least involved the type-1 protease activated receptor (PAR-1). An effect on the fibrinolysis pathway after treatment of HUVECs with thrombin was shown by increased fluorescein-labeled plasminogen binding to cells, which was inhibited by an antibody specific for p11. This was confirmed by observing that thrombin pretreatment of HUVECs increased biotin-modified plasminogen binding. Utilizing a chromogenic assay, pretreatment of HUVECs by thrombin further enhanced activation of the Glu and Lys forms of plasminogen by tPA. These data suggest a novel mechanism that links the coagulation and fibrinolysis pathways by thrombin-mediated feedback.

Key words: Annexin 2, Thrombin, Endothelial cell, Fibrinolysis, Coagulation

Introduction

Annexin 2 (A2) is a Ca²⁺-dependent, anionic phospholipid (aPL)-binding protein that belongs to the ubiquitous multigene annexin family (reviewed by Kim and Hajjar, 2002; Siever and Erickson, 1997; Waisman, 1995). All annexins contain a highly conserved protein core that confers Ca²⁺ and aPL binding, and a unique N-terminus that gives each annexin type-specific properties (Gerke and Moss, 1997; Swairjo and Seaton, 1994). The N-terminus of A2 can associate with p11, an 11 kDa member of the S100 protein family (Glenney et al., 1986; Johnsson et al., 1986; Johnsson et al., 1988). This property allows A2 to coexist as functionally distinct monomeric (A2m) and tetrameric (A2t) forms, the latter resulting from the noncovalent association of two A2m molecules bridged by a p11 dimer (Erikson et al., 1984; Gerke and Weber, 1984; Glenney, 1986). Although both A2m and A2t can bind aPL-containing membranes at millimolar Ca²⁺ concentrations, only A2t is able to bind at intracellular micromolar concentrations (Powell and Glenney, 1987). In addition, both A2m and A2t can aggregate apposing aPL-containing membranes; however, only A2t can subsequently participate in membrane fusion through a complicated and incompletely understood

mechanism (Ali et al., 1989; Drust and Creutz, 1988; Kang et al., 1997; Regnoui et al., 1995).

Differences in the Ca²⁺ requirements for aPL binding of A2m and A2t dictate their intracellular distribution. The ability of A2t to bind aPL at intracellular Ca²⁺ concentrations localizes it to the inner leaflet of the plasma membrane, whereas A2m is found largely in the cytosol (Courtneidge et al., 1983; Osborn et al., 1988; Thiel et al., 1992). An interesting property of A2 cellular distribution is that it has no secretory signal, but has nevertheless been identified on the surface of various cell types (Chung and Erickson, 1994; Hajjar et al., 1994; Kassam et al., 1998; Wright et al., 1995). The mechanism by which A2 is shuttled to the cell surface is not known, but might involve localization to caveolae (Sagot et al., 1997; Stahlhut et al., 2000).

The presence and availability of A2 on the cell surface plays a crucial role in many of its proposed functions. Of relevance to the current study, A2 has been identified as an endothelial cell-surface co-receptor for the fibrinolysis proteins plasminogen and tissue plasminogen activator (tPA) (Hajjar et al., 1994). It has been reported that both A2m and A2t can function as cofactors in the tPA-dependent proteolytic

activation of plasminogen to plasmin (Cesarman et al., 1994; Liu et al., 1995), which proteolytically solubilizes fibrin clots for subsequent clearance. In vivo evidence for an involvement of A2 in fibrinolysis (Rand, 2000) has been demonstrated in acute promyelocytic leukemia, where overexpression of endothelial A2 correlated with accelerated fibrinolysis and bleeding (Menell et al., 1999).

The enzyme directly responsible for fibrin clot production, thrombin, is also a potent cell modulator. Thrombin-mediated activation of cell-surface receptors, including those on endothelial cells, is pleiotropic and results in: increased cytosolic Ca^{2+} , stimulation of various second messenger systems, kinase activation, induction of mitosis, and the flux of ions in and out of the cell (Bartha et al., 1989; Brock and Capasso, 1989; Garcia et al., 1995; Magazine et al., 1996; Molino et al., 1997; Pollock et al., 1988; Schini et al., 1989; Sugama and Malik, 1992; Tesfamariam et al., 1993). Thrombin receptors belong to the protease-activated receptor (PAR) family of seven-transmembrane-domain, G-protein-linked, cell-surface receptors (reviewed by Brass and Molino, 1997; Hou et al., 1998; Jamieson, 1997). To date, three members of the PAR family, PAR-1 (Rasmussen et al., 1991; Vu et al., 1991), PAR-3 (Ishihara et al., 1997) and PAR-4 (Kahn et al., 1998), have been identified as thrombin sensitive. The mechanism of PAR activation is by proteolytic generation of a new N-terminus (Vu et al., 1991), which acts as a 'tethered' receptor ligand. Peptides whose sequences are identical to the first six amino acids of the newly revealed PAR N-terminus have been shown to activate the receptor without a proteolytic step (Garcia et al., 1993; Muramatsu et al., 1992; Tirupathi et al., 1992).

To ensure that the sequential initiation of coagulation and then of fibrinolysis is stringent, feedback communication mechanisms between these pathways have evolved. In the current study, we addressed the hypothesis that an additional mode of communication could be facilitated by the cell-modulating capabilities of thrombin, which might affect activation of plasminogen on human umbilical vein endothelial cells (HUVECs) by altering the availability of cell-surface A2 and p11. The data revealed that thrombin or the analogous thrombin receptor-activating peptide (TRAP) selective for PAR-1 enhances both A2 and p11 on the HUVEC surface, which correlated with increased plasminogen binding and tPA-mediated plasmin generation. The regulation of A2 accessibility on the HUVEC surface is thus a novel variable to be considered in hemostasis.

Materials and Methods

Reagents

Dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), ethylene glycol-bis(ϵ -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), N -2-hydroxyethylpiperazine- N -2-ethanesulfonic acid (Hepes), ϵ -aminocaproic acid (ϵ -ACA), 4',6-diamidino-2-phenylindole (DAPI), bovine serum albumin (BSA), and the thrombin receptor activating peptide (TRAP), SFLLRN, were all from Sigma (Oakville, ON). Recombinant S741C-fluorescein plasminogen (F-Plg), and S741C-biotin plasminogen (B-Plg) were generated as described (Horrevoets et al., 1997). The chromogenic substrate H-D-Val-Leu-Lys- p -nitroaniline dihydrochloride (S-2251) was obtained from Chromogenix (Milano, Italy) Experiments were conducted in either phosphate-buffered saline (PBS; 10.1 mM Na_2HPO_4 , 1.5 mM

KH_2PO_4 , 137.0 mM NaCl, 2.7 mM KCl), Hepes-buffered saline (HBS; 11.0 mM Hepes, 137.0 mM NaCl, 3.0 mM $CaCl_2$, 4.0 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM glucose) or HBS/BSA (HBS supplemented with 1 mg/ml BSA), as indicated.

Antibodies

Several primary antibodies specific for A2m, p11 or vimentin were evaluated to maximize the signal-to-background ratio for cell-surface or intracellular immunofluorescence microscopy, western blot and plasminogen cell-binding analyses. For A2m detection, three different antibodies were found to be best suited for different applications. mAb anti-A2m IgG2a (Oncogene, Cambridge, MA), mAb anti-A2m IgG1 (Transduction Laboratories, San Jose, CA) and mAb anti-A2m IgG1 (Zymed, San Francisco, CA) were used for cell-surface immunofluorescence microscopy, intracellular immunofluorescence microscopy and western blotting, respectively. mAb anti-p11 IgG1 (Transduction Laboratories) was used for both intracellular immunofluorescence microscopy and western blot analyses. The polyclonal rabbit anti-p11 IgG used in plasminogen-binding experiments was a kind gift from D. Waisman (University of Calgary). This antibody was raised to a synthetic peptide corresponding to residues 21-38 of p11 linked to keyhole limpet hemocyanin and affinity purified using the same peptide bound to Sepharose. Polyclonal goat anti-vimentin serum (Sigma) or mAb anti-vimentin IgG1 (Sigma) were used for surface or intracellular detection of antigen by immunofluorescence microscopy, respectively. Species-, subclass- and (where applicable) isotype-matched non-immune negative control antibodies were purchased from Sigma and used at an identical concentration as the specific primary antibodies in every experiment involving antibodies. The secondary antibodies used in immunofluorescence microscopy experiments were fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG (Sigma), FITC-conjugated goat anti-rabbit IgG (Sigma) and Cy³-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, Westgrove, PA). For western blots, purified A2m and p11 provided by D. Waisman were used as positive controls, and mAb anti- β -actin (Sigma) was used to confirm that comparable amounts of cell extract were loaded in each electrophoresis lane. HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) was used as the secondary antibody in all western blotting experiments.

Cell culture

HUVECs were isolated from fresh umbilical cords as previously described (Dudani et al., 1991; Jaffe et al., 1973). Isolated cells were propagated in complete IMDM, consisting of Iscove's Modified Dulbecco's Media (IMDM) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 20 μ g/ml gentamycin (all from GibcoBRL, Burlington, ON), 59 μ g/ml heparin (Sigma) and 10 μ g/ml endothelial cell growth supplement (Calbiochem, Mississauga, ON). All experiments were performed on cells between passage one and five, with no observable differences within this range.

Thrombin and TRAP treatment of cells

HUVECs were grown to confluence on gelatin-coated 22 \times 22 mm glass coverslips or multiwell tissue culture plates. Monolayers were washed once with IMDM/BSA (IMDM supplemented with 1 mg/ml BSA and 2 mM L-glutamine), and treated with the indicated concentrations of α -thrombin (Haematologic Technologies, Burlington, VT) or TRAP in IMDM/BSA for 5 minutes at 37°C. The cells were then washed once with complete IMDM supplemented with 2 mM Ca^{2+} (IMDM/ Ca^{2+}), and further incubated in IMDM/ Ca^{2+} for 1 hour at 37°C. In the absence of the additional hour of incubation, cells were prone to detachment.

Surface A2 and p11 detection

Fluorescence microscopy

To detect cell-surface A2 exclusively, thrombin- or TRAP-treated cells were washed once with complete IMDM, and simultaneously incubated with a purified mAb against A2m (Oncogene; 1.33 µg/ml) and polyclonal goat anti-vimentin serum ([protein]=0.20 mg/ml) for 30 minutes at room temperature (RT). Vimentin is an intracellular cytoskeletal component and was used as a marker for inadvertent permeabilization of cells when surface antigens were being probed. After washing with complete IMDM, cells were incubated with Cy³-conjugated donkey anti-mouse IgG (1.25 µg/ml) and FITC-conjugated rabbit anti-goat IgG (1.66 µg/ml) for 30 minutes at RT. Stained cells were washed extensively and fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in HBS. After fixation, nuclei were stained with DAPI (25 ng/ml) and the cells were washed with PBS and then water prior to mounting on glass slides with 5 µl of mounting media [0.1% phenylenediamine (Sigma), 50% glycerol in PBS].

To visualize the distribution of A2 relative to submembranous cytoskeletal elements, thrombin- or TRAP-treated HUVECs were fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in HBS before immunofluorescent microscopy, as described (Traverso, 2000), to detect A2, vimentin and nuclei simultaneously.

All micrographs were obtained using a Zeiss Axioplan 2 epifluorescent microscope fitted with a Sony 3 CCD color video camera (model DXC-950P). Images were captured by Northern Eclipse (Empix, Mississauga, ON, Canada) software. For all immunofluorescence experiments, individual micrographs presented for comparison were conducted on the same day and subjected to identical image acquisition parameters to optimize observable fluorescence intensity differences between treated and mock-treated cells.

Cell-surface biotinylation

After three washes with HBS, the surface proteins of thrombin- or TRAP-treated HUVECs were biotinylated by incubating the cells for 30 minutes at RT with 0.5 mg/ml of the hydrophilic probe, sulfo-NHS-Biotin (Pierce, Brockville, ON). The washing was repeated, and the cellular proteins were solubilized using Triton X-100 lysis buffer (40 mM TRIS, 150 mM NaCl, 3% Triton X-100, 1 mM EGTA and 0.2 mM PMSF). Surface-labeled proteins were isolated by incubation with avidin-conjugated Sepharose (Sigma) overnight at 4°C. After three washes, the surface proteins bound to the beads were released by the addition of Laemmli sample buffer containing 12.5 mg/ml DTT for 2 minutes at 95°C. To compare the effects of thrombin or TRAP treatment on the amount of surface A2 that is labeled, the affinity-depleted surface proteins were separated by SDS-PAGE (15% polyacrylamide), transferred to polyvinylidene difluoride (PVDF) membranes, and probed with anti-A2 mAb (Zymed; 0.05 ng/ml). After washing, the membranes were incubated with peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch; 0.09 ng/ml), and the bands were detected by chemiluminescence (ECL; Pierce).

The identification of biotinylated surface p11 required an overnight incubation of solubilized cell supernatants at 4°C with non-immune mouse IgG1 (6.7 µg/ml) and then 2 hours at RT with protein A/G Sepharose to reduce nonspecific antibody-protein interactions. After centrifugation, p11 was depleted from clarified supernatants by incubation with anti-p11 mAb (Transduction Laboratories; 6.7 µg/ml) for 2 hours at RT followed by 2 hours with protein A/G Sepharose. As a control for specificity, identically treated cells were incubated with non-immune mIgG1 (6.7 µg/ml). The samples were then subjected to electrophoresis as above for A2. The biotinylated proteins were visualized by probing with horseradish peroxidase conjugated avidin (HRP-avidin) (Sigma; 33.3 ng/ml), followed by ECL detection. To confirm the identity of the observed bands, the blot was subsequently re probed with anti-p11 (0.05 ng/ml).

Intracellular A2 and p11 detection

Fluorescence microscopy

To investigate effects on intracellular A2 and p11, thrombin or TRAP-treated HUVECs were simultaneously fixed and permeabilized with 4% paraformaldehyde, 0.25% glutaraldehyde and 0.2% Triton X-100 prior to incubation with polyclonal goat anti-vimentin serum, anti-A2m mAb (Transduction Laboratories), or anti-p11 mAb (Transduction Laboratories), all at 2.5 µg/ml. The primary antibodies were probed with Cy³-conjugated donkey anti-mouse IgG or FITC-conjugated rabbit anti-goat IgG both at 1.25 µg/ml. Following DAPI treatment, the cells were mounted and visualized as described above.

Western blots

After treatment with thrombin or TRAP, HUVECs grown in tissue culture plates were washed twice with HBS and lysed with Laemmli sample buffer. The proteins were separated by SDS-PAGE under reducing conditions and analyzed by western blotting. A2m and p11 were stained as described for the surface detection of these proteins. As before, the membranes were re probed with anti-actin mAb to ascertain whether constant amounts of cell extract were electrophoresed.

Plasminogen cell binding and activation

Fluorescence microscopy

To visualize the effects of thrombin or TRAP on plasminogen binding to HUVECs, cells were washed with HBS/BSA and incubated with F-P1g (150 nM) for 30 minutes at 37°C in the dark. After three washes with HBS/BSA, the cells were fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in HBS. The nuclear DNA was then stained with DAPI, and the cells were mounted. A requirement for p11 in F-Pg binding to cells was investigated by including polyclonal rabbit anti-p11 antibody (16 µg/ml). As a control for antigen specificity in these experiments, the effects of an identical amount of non-immune rabbit IgG was also evaluated.

Avidin blots

To confirm the F-Pg experiments, after washing once with HBS/BSA, thrombin- or TRAP-treated cells were incubated with B-P1g (150 nM) in HBS/BSA for 30 minutes at 37°C. Following the removal of unbound B-Pg by washing, the clarified cell extracts were subjected to SDS-PAGE (10%). Biotinylated surface p11 was detected as above with avidin-HRP. In each case, actin was used as a control for consistency in the amount of cell extract loaded per lane. Identical experiments were conducted with either unlabeled Glu-plasminogen (1.5 µM) or ε-ACA (3 mM) being added to the B-P1g solutions as competitive inhibitors.

Chromogenic assay

HUVECs grown to confluence in 48-well culture plates were treated with 8 nM thrombin or 10 µM TRAP as described above. After recovery in IMDM/Ca²⁺ for 30 minutes at 37°C, the cells were further incubated with 5 nM recombinant tPA in HBS/BSA for 20 minutes. Glu- or Lys-plasminogen (150 nM) was then added to the reaction mixture. Aliquots were removed at various time points, and the amount of plasmin generated was measured by cleavage of the chromogenic substrate S-2251. To control for cellular tPA production or the presence of plasminogen in the culture media, the experiment was repeated without exogenous tPA, plasminogen, or both. The experiment was furthermore performed in the presence of the protease inhibitor aprotinin [100 KIU (Kallikrein inactivator units)], to confirm that the chromogenic activity observed was attenuated by a known plasmin inhibitor.

Results

HUVEC surface A2 and p11 are enhanced by thrombin or TRAP

In all attempts to surface-stain HUVECs where fixation in the absence of detergent preceded probing with antibodies, small vimentin patches were visible. Therefore, to ensure the exclusive detection of surface antigens, fixation prior to addition of antibodies was avoided. Fig. 1 shows the effect of thrombin or TRAP on the amount of surface A2. Immunofluorescent staining of live, unfixed HUVECs confirmed previous reports of endogenous A2 on the surface of unstimulated HUVECs. However, following thrombin pretreatment, the amount of A2 associated with the cell surface was observed to have increased several fold. Vimentin, an intermediate filament of the cytoskeleton, was not visible and micrographs were devoid of its characteristic intracellular spindle pattern, demonstrating that the cells being studied were not permeabilized. By this criterion, the A2 observed was due exclusively to the accessible cell-surface pool. To confirm dependence of the fluorescent signal on the primary antibody, controls using isotype-matched non-immune antibody in place of anti-A2 on identically treated cells were negative (data not shown). Nuclear staining with DAPI verified that a similar number of cells were in each field of view. Under identical conditions, TRAP pretreatment of HUVECs resulted in a comparable increase in the amount of surface A2 as observed following thrombin treatment. Since TRAP selectively triggers cell activation through PAR-1, these data demonstrate the specificity of thrombin and the fact that, of the three known thrombin-sensitive receptors, at least PAR-1 is involved.

The finding that staining unfixed, native HUVECs must be conducted to ensure the exclusive detection of surface A2 by immunofluorescence microscopy, excluded using the p11 antibodies we evaluated. These gave an unacceptable signal-to-background ratio when cells were not fixed. The exposure of HUVEC surface p11 was consequently probed by chemically modifying native cells with a hydrophilic amine-reactive derivative of biotin that cannot cross the plasma membrane into the cell, as previously described (Kassam et al., 1998). These experiments confirmed that HUVEC surface A2m was increased by pretreatment of the cells with thrombin or TRAP (Fig. 2). Furthermore, p11 was increased compared with mock-treated cells, suggesting that A2t may be shuttled to the HUVEC surface after cell stimulation by thrombin or TRAP. The identity of the biotinylated proteins was determined antigenically and by electrophoretic migration compared with purified A2 or p11 (data not shown).

Thrombin or TRAP enhance total HUVEC p11

As shown in Fig. 3A, the effects of thrombin or TRAP on intracellular amounts of A2 and p11 were evaluated by immunofluorescence microscopy of fixed and detergent-permeabilized HUVECs. When HUVECs were pretreated with thrombin or TRAP, the detection of p11 antigen was enhanced compared with mock-treated cells. By contrast, the amount of A2 detected was not changed. No obvious difference in the distribution of intracellular A2 was observed by this method. Identically treated cells were stained for vimentin to ensure all cells were equally permeabilized, and that thrombin or TRAP did not affect the extent of permeabilization. The expected

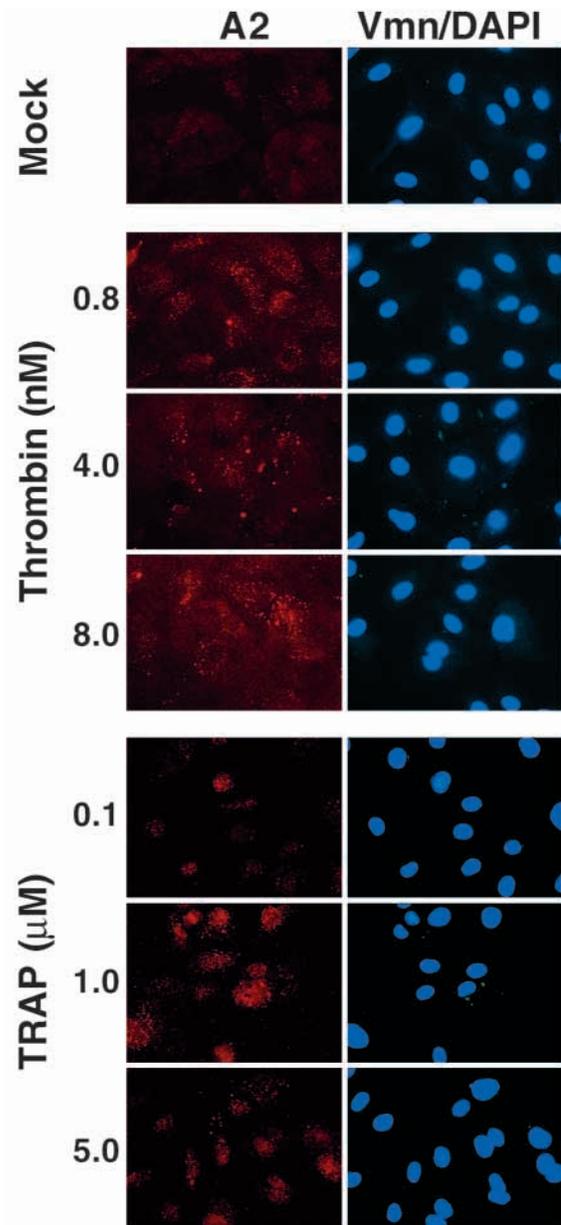


Fig. 1. Immunofluorescent detection of surface A2 on HUVECs. HUVECs grown on gelatin-coated glass coverslips were treated for 5 minutes with either thrombin or TRAP and allowed to recover for 1 hour. The cells were then simultaneously stained under native conditions for annexin 2 (A2; red) and the intracellular marker vimentin (Vmn; green) followed by fixation. The number and position of individual cells were determined by detection of nuclei using DAPI (blue).

spindle pattern for vimentin was seen for each cell. In addition, the corresponding isotype controls for thrombin- or TRAP-treated cells were negative (data not shown), indicating that the observed A2, p11 and vimentin staining required the respective specific primary antibody. Visualization of nuclei with DAPI confirmed that a similar number of cells per field were being evaluated.

To corroborate the effects of thrombin or TRAP on

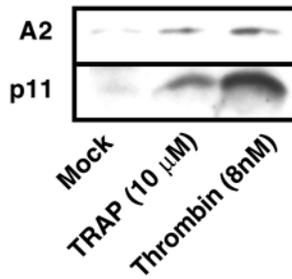


Fig. 2. Detection of HUVEC surface A2 and p11 by biotinylation. Following treatment with thrombin or TRAP, HUVECs were biotinylated using membrane-impermeable sulfo-NHS-biotin. Surface-labeled proteins were affinity depleted using avidin-Sepharose and probed by western blotting using anti-A2 mAb, while p11 was immuno-depleted and detected using HRP-avidin.

intracellular A2 or p11 observed by immunofluorescence microscopy, western blot analyses were conducted (Fig. 3B). This experiment also showed that both stimuli enhanced the amount of intracellular p11, while A2 levels remained unchanged. The PVDF membranes were subsequently reprobed for the cytoskeletal protein β -actin, confirming that the same amount of cell extract was being loaded in each lane.

Thrombin or TRAP enhance submembranous A2

To determine if A2 colocalizes with submembranous cytoskeletal patches as previously described for annexin I (Traverso et al., 1998), HUVECs were fixed with paraformaldehyde and glutaraldehyde in the absence of detergent. These 'pre-fixed' cells were simultaneously stained for A2, vimentin and nuclear material, and evaluated by fluorescence microscopy. Fig. 4 demonstrates that this fixing protocol results in an obviously different distribution of vimentin and A2 compared with the surface staining of unfixed cells (Fig. 1). Here, patches of vimentin were observed to colocalize with A2, which is highlighted by overlaying the micrographs (Fig. 4). The absence of typical vimentin spindles seen for detergent-permeabilized cells (Fig. 3) demonstrated that typical permeabilization did not occur and was consistent with the previous well-established conclusion that these represent submembranous domains (Traverso et al., 1998). After treatment with thrombin or TRAP, the number and extent of these locales of vimentin and A2 were increased (Fig. 4). Isotype controls conducted on identically treated cells confirmed that all staining observed was dependent on specific antibody-antigen interactions (data not shown).

Enhancement of plasminogen binding to thrombin- or TRAP-treated HUVECs

A2 has been shown to accelerate plasmin generation by functioning as a cell-surface coreceptor for plasminogen and tPA (Hajjar et al., 1994; Kassam et al., 1998). To determine whether thrombin- or TRAP-induced enhancement of surface A2 and/or p11 could play a role in plasmin generation, we studied their effects on the binding of fluorescein (F-Plg)- or biotin (B-Plg)-labeled recombinant S741C-plasminogen. After thrombin or TRAP treatment of HUVECs (Fig. 5A), F-Pg

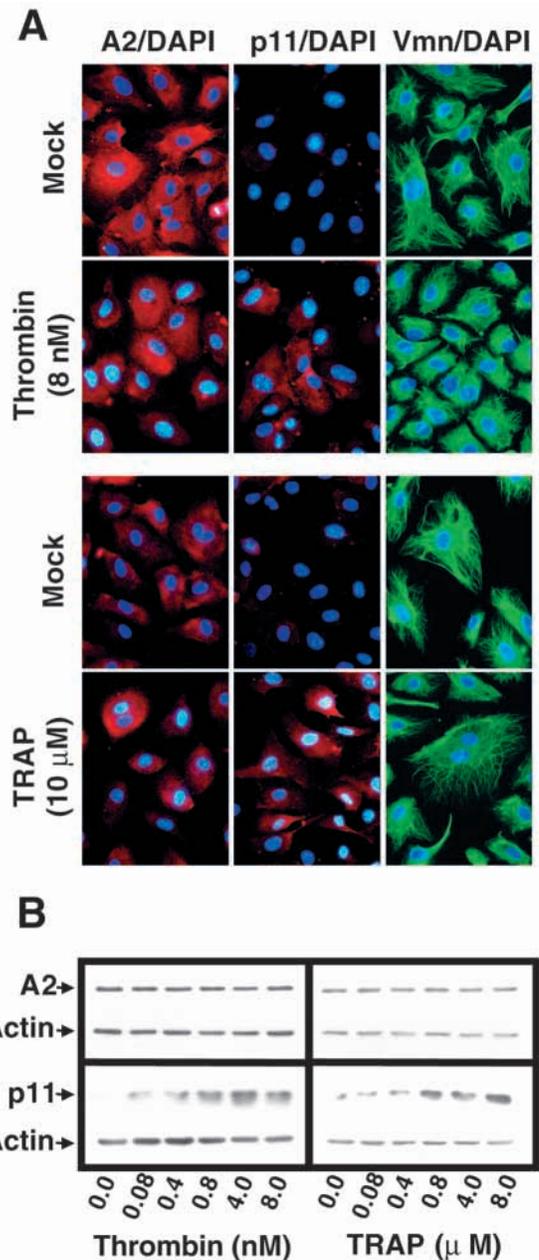


Fig. 3. Detection of A2 and p11 in permeabilized HUVECs. HUVECs were treated with thrombin or TRAP for 5 minutes at 37°C, washed once with culture media supplemented with 2 mM Ca^{2+} , and allowed to recover for 1 hour at 37°C. After recovery, cells grown on glass coverslips (A) were detergent permeabilized, fixed and stained for A2, p11 or vimentin (Vmn) and DAPI, whereas those grown in multiwell plates (B) were subjected to western blot analysis.

binding followed by fluorescence microscopy was enhanced several fold compared with the mock-treated cells. To correlate the increase in F-Plg binding to p11, a rabbit anti-p11 polyclonal antibody was able to inhibit completely the enhancement of F-Plg binding observed. At comparable concentrations of non-immune rabbit IgG, or other p11 or A2m antibodies, F-Pg binding to the treated cells was not affected (data not shown). Staining of nuclei with DAPI demonstrated

that similar numbers of cells were being evaluated in each field. Since these inhibition experiments were performed using the conditions we established for detection of surface antigens on HUVECs, they further support the presence of p11 on the stimulated HUVEC surface.

To confirm the F-Plg fluorescence microscopy experiments, binding of B-Plg to cells treated with thrombin or TRAP was evaluated by HRP-avidin blots (Fig. 5B). We observed that either thrombin or TRAP enhanced B-Plg binding to HUVECs compared with mock-treated cells. The addition of ϵ -ACA, which inhibits the interaction of plasminogen with essential C-terminal lysines on known receptors (Miles et al., 1991), or a tenfold excess of unlabeled purified Glu-plasminogen completely inhibited B-Plg binding (data not shown). Reprobing the PVDF membrane for β -actin served as a loading

control and verified that comparable amounts of cell extract were subjected to electrophoresis.

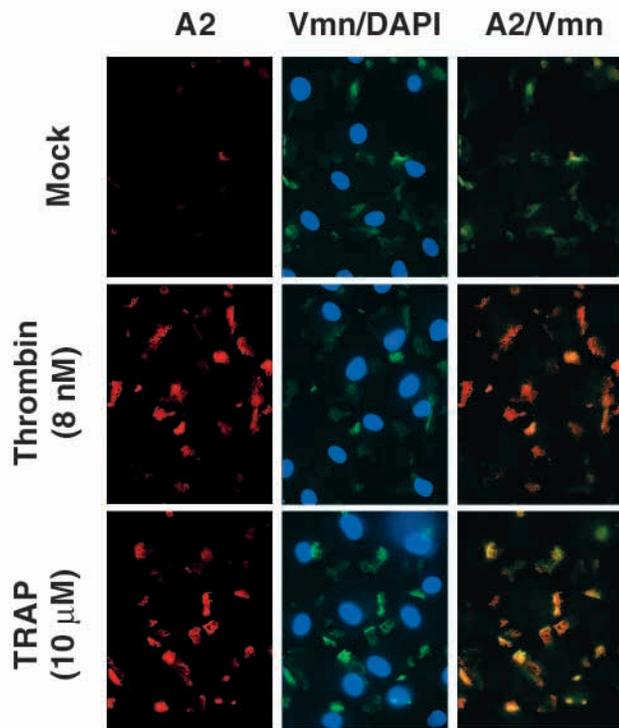
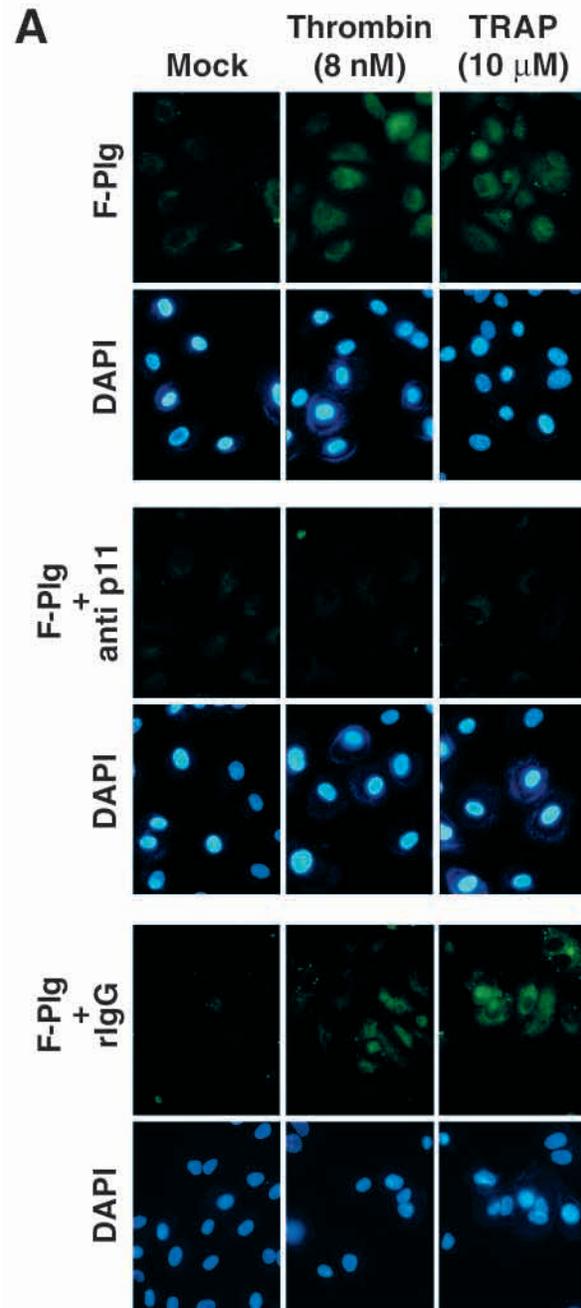
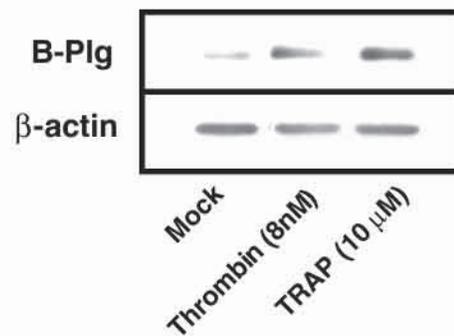


Fig. 4. Immunofluorescent detection of submembranous A2 in HUVECs. Following treatment for 5 minutes with thrombin or TRAP and recovery for 1 hour, HUVECs were fixed in the absence of detergent and stained simultaneously for A2, vimentin (Vmn) and nuclei (DAPI).

Fig. 5. Effect of thrombin or TRAP on plasminogen binding to HUVECs. (A) Fluorescein-plasminogen (F-Plg) binding to HUVECs was evaluated following thrombin or TRAP treatment by incubating the cells with F-Plg for 30 minutes at 37°C. The cells were subsequently fixed, and DNA was stained with DAPI. Identical experiments were performed in which both media and F-Plg solutions contained either polyclonal anti-p11 IgG or non-immune rabbit IgG (rIgG). (B) Biotinylated plasminogen (B-Plg) binding to the surface of thrombin- or TRAP-treated HUVECs was evaluated by incubating cells with B-Plg for 30 minutes at 37°C. After washing unbound ligand, the cells were lysed and subjected to blot analysis using HRP-avidin. To ensure identical amounts of cell lysate were loaded in each lane, the blots were reprobed with anti- β -actin antibody.



B



Plasmin generation on HUVECs is enhanced by thrombin

Having established that thrombin-mediated stimulation of HUVECs can enhance plasminogen binding, we investigated whether the effects translated to increased plasmin generation on the HUVEC surface. tPA-mediated activation of the Lys (Fig. 6A) or Glu forms of plasminogen to plasmin and was measured using a chromogenic substrate specific for plasmin (Fig. 6B). These experiments demonstrated a significant acceleration of plasmin generation on the HUVEC surface due to prior treatment with thrombin. The omission of tPA or plasminogen prevented generation of chromogenic activity, indicating that the cells were not secreting detectable amounts of tPA, and that plasmin generation was dependent solely on exogenous tPA and plasminogen (data not shown). The addition of aprotinin, an effective inhibitor of plasmin, blocked all chromogenic activity observed (data not shown), a further indicator that the assay was specific for plasmin generation.

Discussion

Thrombin is a well-known cell stimulus that functions to feedback-regulate hemostasis. In the current study, we

identified a novel effect of thrombin on the cellular pathway that controls production of the fibrinolytic enzyme, plasmin. The hypothesis addressed was that thrombin-mediated cell signaling augments endothelial cell participation in plasmin generation by enhancing cell-surface A2, a known tPA cofactor. Since the majority of A2 is intracellular (Hajjar et al., 1996), a procedure was developed to ensure that surface A2 was exclusively visualized by immunofluorescence microscopy. To serve as a control for accidental permeabilization of the cell membrane, an intracellular cytoskeletal component, vimentin, was stained simultaneously along with A2. By this criterion, selective detection of surface A2 required that antibodies be incubated with HUVECs that had not been subjected to prior fixing with glutaraldehyde or paraformaldehyde. No vimentin was visible by this method, which enabled us to conclude that A2 was indeed increased on the surface of HUVECs within 1 hour of thrombin treatment.

Using identical conditions that were found by immunofluorescence microscopy to ensure no inadvertent permeabilization of cells, surface biotinylation of HUVECs confirmed that thrombin treatment resulted in an increase to the amount of A2 on the HUVEC surface. Furthermore, this method enabled us to conclude that HUVEC surface p11 was concomitantly increased due to thrombin-mediated effects. Since A2 is required to anchor p11 to the cell membrane (Zobiack et al., 2001), our observation of enhanced p11 implies that the tetrameric form of A2 (i.e. A2t) is being increased on the cell surface by thrombin.

Thrombin triggers transmembrane signaling through proteolytic stimulation of the PAR family of cell-surface receptors (Coughlin, 2001). To determine whether PAR-1 participates in the effects of thrombin that we observed on A2 surface exposure, a well-characterized peptide corresponding exclusively to the PAR-1 tethered receptor ligand, SFLLRN (TRAP), was used to treat HUVECs instead of thrombin. By immunofluorescence or surface biotinylation of TRAP-treated cells, we found an enhancement of A2 and p11 on the HUVEC surface, demonstrating that at least PAR-1 is involved.

To determine whether thrombin-mediated translocation of A2 and p11 to the cell surface was concurrent with antigenic changes inside of cells, immunofluorescence microscopy of fixed and permeabilized cells and western analysis of total cell extracts were conducted. These experiments revealed that, whereas thrombin has no effect on the amount of A2, p11 is increased significantly. This finding suggests that levels of intracellular p11 might control the extent of transmembrane trafficking.

Neither A2 nor p11 contain secretory signals, therefore the finding of either on the cell surface is intriguing since the mechanism by which they are trafficked to the cell surface is not known and cannot involve the typical endoplasmic reticulum-dependent pathway (Siever and Erickson, 1997). The observations presented here document the only known trigger for redistribution of A2 and p11 to the HUVEC exterior and provide insight into the mechanism since thrombin induces signaling through G proteins. Like thrombin, the effects of nicotine (Zheng et al., 1997) and nerve growth factor (Rakhit et al., 2001) on certain cells have been suggested to involve functions of G proteins. Combining the current thrombin studies with reports that: (1) nicotine stimulation of chromaffin cells causes translocation of A2 from the cytosol to caveolar

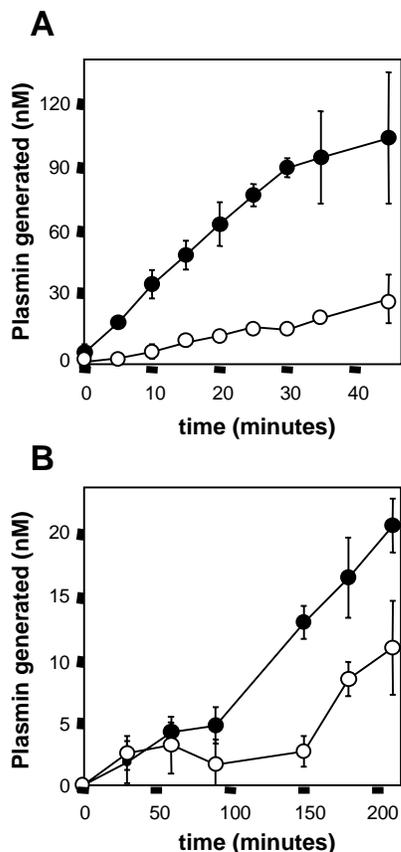


Fig. 6. Effect of thrombin on HUVEC-dependent plasmin generation. Following mock (○) or 8 nM thrombin (●) treatment, HUVECs were incubated with 5 nM recombinant tPA for 20 minutes at 37°C. Lys-plasminogen (A) or Glu-plasminogen (B) were then added at a concentration of 150 nM, aliquots were removed at the indicated times and assayed for plasmin activity by chromogenic substrate (S2251) reactivity ($n=3$, \pm s.d.).

domains (Sagot et al., 1997); (2) A2 has been identified as a component of caveolae (Stahlhut et al., 2000; Harder and Gerke, 1994; Stan et al., 1997); (3) cell-surface A2 has been colocalized with caveolin (van der Goot, 1997); and (4) nerve growth factor induces exposure of A2 on the neurite surface (Jacovina et al., 2001), we propose that A2 transport to the cell surface may involve G-protein-linked control of caveolae.

To add credence to the hypothesis that submembranous structures may be involved in the transmembrane trafficking of A2, we utilized a cell-fixation method in the absence of detergent that previously enabled identification by electron and immunofluorescence microscopy of distinct regions proposed to occur at junctions between the cytoskeleton and the plasma membrane (Traverso et al., 1998). This fixation method showed that thrombin stimulation enhances the HUVEC submembranous A2, which colocalized with the intermediate cytoskeletal component vimentin. The thrombin-mediated increase in intracellular p11 we observed might in part explain the elevation of submembranous A2 by facilitating A2t formation and consequent shuttling to the submembrane, as demonstrated recently for HepG2 cells (Zobiack et al., 2001). The probability that submembranous A2t would be preferentially translocated to the extracellular surface compared with cytosolic A2m helps to rationalize the previous conclusion that A2t represents much of the cell-surface A2 (Kassam et al., 1998).

A2 is recognized as an endothelial cell-surface coreceptor for tPA and plasminogen, and a cofactor in the tPA-dependent conversion of plasminogen to plasmin. Although the ability of A2 to bind plasminogen is well documented, there is controversy in the literature over whether A2m (Cesarman et al., 1994; Hajjar et al., 1994; Hajjar et al., 1998; Kang et al., 1999) or A2t (Choi et al., 1998; Liu et al., 1995) is the functional form on the HUVEC surface. Our observations that both A2 and p11 antigen were increased on the HUVEC surface after thrombin treatment led us to test whether this effect correlated to enhanced fibrinolytic cell-surface activity. As predicted, greater binding of fluorescein- or biotin-labeled plasminogen to the cell surface was observed after thrombin or TRAP-mediated HUVEC stimulation. Like previous experiments with purified A2 (Cesarman et al., 1994; Kang et al., 1999), we found ϵ -ACA inhibited plasminogen binding to HUVECs. Because thrombin might enhance numerous putative plasminogen receptors on the cell surface, immuno-inhibition was conducted. Our observation that polyclonal anti-p11 antibody and not non-immune control antibody inhibited the thrombin- or TRAP-mediated increase in F-PIg binding confirmed the importance of p11. Although we were unable to identify an antibody specific for A2 that was inhibitory (data not shown), we cannot exclude the possible simultaneous direct involvement of A2m or the A2 subunit of A2t. However, a role for p11 is strongly supported to explain our additional finding that thrombin pretreatment of HUVECs enhances tPA-dependent plasmin generation on the cell surface.

Cumulatively, the data presented here are consistent with a model where stimulation of HUVECs by thrombin enhances the accessibility of A2 and p11 on the cell surface. Once on the surface of the cell, at least the p11 subunit of A2t functions as a plasminogen receptor to accelerate plasmin generation by tPA. Thus, a novel feedback regulatory step in hemostasis is indicated that links thrombin, the biological mediator of

coagulation, to enhanced cellular production of the fibrinolytic enzyme, plasmin. Cell-surface A2 has been suggested to have importance in several processes, such as tenascin C-binding (Chung and Erickson, 1994), tumor invasion (Mai et al., 2000) and cytomegalovirus infection (Raynor et al., 1999; Wright et al., 1995). Therefore, the finding that exposure of cell-surface A2 can be induced by thrombin might have implications in areas additional to fibrinolysis.

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