Human Neutrophil Elastase Proteolytically Activates the Platelet Integrin \( \alpha_{IIb}\beta_3 \) through Cleavage of the Carboxyl Terminus of the \( \alpha_{IIb} \) Subunit Heavy Chain

INVOLVEMENT IN THE POTENTIATION OF PLATELET AGGREGATION*

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Neutrophil elastase (NE) and cathepsin G are two serine proteinases released concomitantly by stimulated polymorphonuclear neutrophils. We previously demonstrated that while NE by itself does not activate human platelets, it strongly enhances the weak aggregation illustrated that while NE by itself does not activate human polymorphonuclear neutrophils. We previously delineated the molecular mechanisms involved in this potentiation process. Two main pieces of data prompted us to focus on the activation of the platelet fibrinogen receptor, the \( \alpha_{IIb}\beta_3 \) integrin. First, previous studies have shown this integrin to be particularly prone to proteolytic regulation of its function. Second, we found that the potentiating activity of NE on the threshold of cathepsin G-induced platelet aggregation was strictly dependent on the presence of exogenous fibrinogen. Using flow cytometry analysis, NE was shown to trigger a time-dependent binding of PAC-1 and AP-5, two monoclonal antibodies specific for the activated and ligand-occupied conformers of \( \alpha_{IIb}\beta_3 \). Furthermore, the potency of NE was shown to result from an increased capacity of platelets to bind fibrinogen. Indeed, the combination of NE and threshold of cathepsin G increased the binding of PAC-1 \(~5.5\)-fold over basal values measured on nontreated platelets, whereas this binding was only by \(~3\)-fold in threshold of cathepsin G-stimulated platelets (\( p < 0.05 \)). By contrast, phosphatidic acid accumulation, pleckstrin phosphorylation, and calcium mobilization produced by the combination of NE and threshold of cathepsin G were not significantly different from those measured with threshold of cathepsin G alone (\( p > 0.05 \)), indicating that the phospholipase C/protein kinase C pathway is not involved in the potentiation of aggregation. The foregoing data, as well as the requirement of catalytically active NE to trigger \( \alpha_{IIb}\beta_3 \) activation and potentiate threshold of cathepsin G-initiated platelet aggregation, led us to examine whether the structure of this integrin was affected by NE. Immunoblot and flow cytometry analysis revealed a limited proteolysis of the carboxyl terminus of the \( \alpha_{IIb} \) subunit heavy chain (\( \alpha_{IIbH} \), as judged by the disappearance of the epitope for the monoclonal antibody PMI-1. Mass spectrometry studies performed on a synthetic peptide mapping over the cleavage domain of \( \alpha_{IIbH} \) predicted the site of proteolysis as located between Val\( ^{330} \) and Asp\( ^{332} \). Treatment by NE of ATP-depleted platelets or Chinese hamster ovary cells expressing human recombinant \( \alpha_{IIb}\beta_3 \) clearly established that activation of the integrin was independent of signal transduction events and was concomitant with the proteolysis of \( \alpha_{IIbH} \). In support of this latter observation, a close correlation was observed between the kinetics of proteolysis of \( \alpha_{IIbH} \) on platelets and that of expression of the ligand binding activity of \( \alpha_{IIb}\beta_3 \) (\( r^2 = 0.902, p = 0.005 \)). However, only a subpopulation (\(~25\%) of the proteolyzed \( \alpha_{IIb}\beta_3 \) appeared to fully express the ligand binding capacity. Altogether, these results demonstrate that NE up-regulates the fibrinogen binding activity of \( \alpha_{IIb}\beta_3 \) through a restricted proteolysis of the \( \alpha_{IIb} \) subunit, and that this process is relevant for the potentiation of platelet aggregation.

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Thrombosis and inflammation are processes which result from complex relationships between various vascular cell types, *i.e.* endothelial cells, leukocytes, and platelets (1, 2). As part of such a cell cooperation network, polymorphonuclear neutrophils contribute to vessel injury not only by their own, but also through interactions with platelets. Thus, neutrophils are found admixed with platelets in the core of vascular occlusions in several experimental models (1, 3), and more importantly, a neutrophil-dependent platelet deposition has been described in arterial injuries (4–6). Neutrophil-mediated platelet activation can be demonstrated in *vitro* by adding specific neutrophil agonists such as the formyl-Met-Leu-Phe (*fMLP*) peptide, tumor necrosis factor-\( \alpha \), or interleukin-8 to autologous neutrophil-platelet mixed suspensions (7–10). Cathepsin G, a serine proteinase stored in the azurophilic granules of neutrophils and released upon their stimulation, has been established as the major mediator of this cell-to-cell interaction (11–13). Acting similarly to \( \alpha \)-thrombin, another serine proteinase agonist of platelets, cathepsin G-induced platelet activation results in massive exocytosis and aggregation reactions. The
potent signal transduction triggered by this neutrophil proteinase includes the activation of an as yet unidentified proteinase-activated membrane receptor, and the subsequent stimulation of the phospholipase C (PLC) 1-protein kinase C (PKC) and Ca\(^{2+}\) pathways (14–16). Another important serine proteinase released from the azurophilic granules concomitantly with cathepsin G is neutrophil elastase (NE). While NE fails to trigger platelet aggregation and exocytosis (17–19), it has been demonstrated that when cathepsin G and NE are added together at concentrations comparable to those released by FMLP-activated neutrophils, NE potentiates the capacity of platelets to aggregate in response to cathepsin G (18, 19). However, the molecular mechanism underlying this synergism remains unknown.

Platelet aggregation is primarily mediated by the binding of the bifunctional adhesive protein fibrinogen to the surface of adjacent activated platelets, and considerable evidence has established the integrin \(\alpha_{IIb}\beta_3\) (glycoprotein Iib-IIIa) as the membrane receptor for fibrinogen, thus supporting platelet aggregation (for review, see Refs. 20–22). As all other integrin membrane receptors for fibrinogen, thus supporting platelet aggregation (for review, see Refs. 20–22), \(\alpha_{IIb}\beta_3\) is a transmembrane receptor with a single transmembrane domain, and presents a complex pattern of intramolecular disulfide bonds within its large extracellular domain (20). Although \(\alpha_{IIb}\beta_3\) is a constitutively expressed on the platelet plasma membrane, the receptor normally acquires its capacity to bind fibrinogen only upon platelet activation. An inside-out signaling process is likely responsible for converting this integrin from a low-affinity to a high-affinity membrane receptor for fibrinogen, through conformational modifications of its extracellular domains (21, 22). During platelet exocytosis, translocation to the plasma membrane of the fraction of \(\alpha_{IIb}\beta_3\) complexes associated with the internal \(\alpha\)-granules (24) is another mean for increasing the capacity of activated platelets to bind fibrinogen (25). Finally, an alternative pathway for activation of \(\alpha_{IIb}\beta_3\) at the surface of platelets could be a proteolytic modification of the extracellular regions of this receptor. Thus, exposure of platelets to pancreatic or leukocyte elastases (26, 27) or to \(\alpha\)-chymotrypsin (28–30) has been reported to induce the irreversible expression of fibrinogen-binding sites in the absence of intracellular activation.

In view of these data, and considering the potential physiopathological importance of the process, the aim of the present study was to delineate the molecular mechanism(s) involved in the potentiation exerted by NE on cathepsin G-induced platelet aggregation. For this purpose, we considered both the possible involvement of intracellular signaling pathways, and the changes in the structure and biological activity of the \(\alpha_{IIb}\beta_3\) integrin brought about by NE alone or in combination with cathepsin G.

### MATERIALS AND METHODS

#### Antibodies and Reagents

Except for the monoclonal antibody PAC-1, which was provided by the University Cell Center of Pennsylvania (Philadelphia, PA), the murine monoclonal and rabbit polyclonal domain-specific anti-\(\alpha_{IIb}\beta_3\) antibodies used in this study and listed in Table I were obtained from the Scripps Research Institute (La Jolla, CA): FMI-1 and the polyclonal antisera against the peptide V41 (designated anti-V41) were kindly supplied by Dr. M. H. Ginsberg, AP-2 and AP-5 were generous gifts from Dr. T. J. Kunicki, and the polyclonal antisera IIb10 was kindly provided by Dr. S. E. D’Souza. Rabbit polyclonal antisera against purified SDS-denatured whole \(\alpha_{IIb}\) or \(\beta_3\) (designated as anti-\(\alpha_{IIb}\) and anti-\(\beta_3\)) have been previously described and characterized (30). Negative control IgG or IgM isotype antibodies were from Sigma and DAKO (Glostrup, Denmark), respectively. Fluorescein isothiocyanate-conjugated anti-IgG or anti-IgM were obtained from DAKO and Sigma, respectively. Reagents for SDS-PAGE were from Bio-Rad. Nitrocellulose membranes (0.45 \(\mu\)m pores) were from Schleicher and Schuell (Dassel, Germany). Affinity-purified staphylococcal 125I-protein A and carrier-free sodium [125I]iodide were from Amersham International plc (Little Chalfont, United Kingdom). The PKC inhibitor GF 109203X was a kind gift from Dr. J. Kirilovsky (Laboratoire Glaxo-Wellcome, Les Ulis, France). This compound was dissolved in Me2SO (which final concentration in platelets was less than 0.5%, v/v). Eglin C was generously provided by Dr. H. P. Schnebi (Ciba-Geigy Research, Basel, Switzerland). Blood was obtained from the Centre National de Transfusion Sanguine (Paris, France). Fibrinogen (Grade L) was purchased from Kabi (Stockholm, Sweden) and treated with diisopropyl fluorophosphate to activate coagulant contaminants and then dialyzed to remove the free inhibitor. N-Succinyl-(Ala)\(_2\),Pro-Phe-p-nitroanilide (a cathepsin G substrate), N-succinyl-(Ala)\(_3\),p-nitroanilide (a NE substrate), 2-deoxy-d-glucose, sodium azide (NaN\(_3\)), glucono-d-lactone, the proteinase inhibitors phenylmethylsulfonyl fluoride (PMSF), benzamidine, leupeptin, soybean trypsin inhibitor and aprotinin were from Sigma. Iscove’s buffer was from BioWhittaker (Belgium). The peptide FPPQPVNLKVDWG (the single-letter code for amino acids), corresponding to the sequence 827–841 of the \(\alpha_{IIb}\) subunit heavy chain, was synthesized by Neosystem Labortoires (Strasbourg, France). All other reagents were obtained as indicated in Si-Tahar et al. (16).

#### Purification of Neutrophil Cathepsin G and NE

Cathepsin G and NE were purified as described previously (16), using a two-step chromatographic procedure (aprotinin-Sepharose affinity and CM-Trisacryl hydrophobic interaction). The neutrophil proteinases was assayed by SDS-PAGE. Moreover, it was verified that cathepsin G and NE preparations were devoid of each others proteinase by monitoring spectrophotometrically the hydrolysis of N-succinyl-(Ala)\(_2\),Pro-Phe-p-nitroanilide and N-succinyl-(Ala)\(_3\),p-nitroanilide induced by purified NE and cathepsin G, respectively. For the determination of their active site concentrations, a constant amount of the enzyme was reacted in the presence of increasing concentrations of \(\alpha\)-antitrypsin and extrapolation of the concentrations were performed using linear regression analysis.

To block the catalytic site of NE, the purified proteinase (70 \(\mu\)g) was incubated for 60 min at 25 °C with PMSF (1.25 \(\mu\)M) and the mixture was subsequently dialyzed to remove the free inhibitor. PMSF-treated NE was shown to be proteolytically inactive by testing the lack of hydrolysis of its specific synthetic substrate.

#### Preparation and Labeling of Platelets

Blood was obtained from healthy adult volunteers without any medication. The platelet-rich plasma was isolated by centrifugation of blood at 180 \(\times\) g for 20 min and incubated with 5\(^{-}\text{[3C]}\text{HT}\) (0.05 mCi/ml) for 30 min at 37 °C. For protein phosphorylation and phospholipid metabolism studies, platelets were labeled with \(^{32}\text{P}\)orthophosphoric acid as described previously (6). Then, labeled platelets were washed by two successive centrifugations (1.600 \(\times\) g, 10 min) and resuspended in Tyrode’s buffer such that the final platelet concentration was 4 \(\times\) 10\(^{11}\) cells/ml. The entire procedure was performed at 37 °C and isolated platelets were maintained at this temperature until use.

#### Generation of Stable CHO Cells Expressing Human \(\alpha_{IIb}\beta_3\) (CHO/\(\alpha_{IIb}\beta_3\))

The full-length cDNA encoding wild type human \(\alpha_{IIb}\) (35) or human \(\beta_3\) (36) were inserted into the pBEJ expression vector and cotransfected into CHO dhfr(NEG) cells as described previously (37). Briefly, 20 \(\mu\)g of

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1 The abbreviations used are: PLC, phospholipase C; NE, neutrophil elastase; threshold of cathepsin G, threshold concentration of cathepsin G; PKC, protein kinase C; P47, pleckstrin; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; LIBS, ligand-induced binding site; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PGI\(_1\), prostaglandin I\(_2\); PdOH, phosphatidic acid; 5-HT, 5-hydroxytryptamine.
each αthb and βth cDNA and 2 μg of dihydrofibrate reductase plasmid (pMGR901) were mixed with 40 μg of LipoAMINE in a final volume of 200 μl and added to the cells. After a 48-h incubation of the cells in Iscove's medium supplemented with 10% heat-inactivated fetal calf serum, the cells were grown in nucleoside-free α-minimal essential medium or when necessary ibuprofen was used as selective medium. Positive transfectants were selected for cell surface expression of recombinant αthb using the complex specific anti-αthb monoclonal antibody AP-2 (32) and goat anti-mouse IgG-coated immunomagnetic beads. Cells were further grown to confluence in T75 or T25 flasks in Iscove's buffer supplemented with glutamine, penicillin, streptomycin, and 10% fetal calf serum and passed after hypertonic chloroform. Phosphoinositides and phosphatidic acid (PtdOH) were separated by Sepharose chromatography, and labeled with 125I to a specific activity of 4.5 × 104 becquerels/μg of IgG using the chloramine T procedure (32, 41). Platelets were exposed to NE (400 nM) in an aggregometer cuvette with a 25 μl of a stopping solution made of 77 mM EDTA, 155 mM NaCl, 35 mM formaldehyde (0.85% v/v), or 1.8 μl of isometric chloroform, methanol, 12 M HCl, 0.1 mM EDTA (20:40:1:2, v/v), respectively, to terminate the reaction. Supernatants containing released 5-125I-chloroform were mixed with scintillation fluid for measuring radioactivity. Aggregation was expressed as the percent of change in light transmission and the 5-125I-chloroform platelet content.

**Protein Phosphorylation and Polyphosphoinositide Metabolism**

Chloroform/distilled water (0.5 volume of each) was added to 35P-labeled platelets diluted in the stopping organic solution (see above). This suspension was vigorously shaken and centrifuged for 10 min at 10 °C. The upper aqueous phase was discarded. Proteins, concentrated at the interface, were solubilized according to the procedure of Laemmli (38). Radiolabeled proteins were then subjected to SDS-PAGE using a 12.5% resolving gel and a 5% stacking gel. After staining, dried gels were exposed to a Molecular Dynamics (MD; Envry, France) Phosphor-Imaging screen. Concurrently, the lower chloroformic phase was evaporated, washed according to Jolles et al. (39), and resuspended in chloroform. Phosphoinositides and phosphatidic acid (PtdOH) were separated by thin layer chromatography using chloroform/acetone/methanol/n-butanol/0.15 M KH2PO4 (20:40:12:17, v/v) as the migration solvent. Upon drying, the chromatography plates were also exposed to a PhosphorImaging screen. PLC and PKC activities were evaluated by quantifying the radioactive signals associated to PtdOH and pleckstrin, respectively, to terminate the reaction. Supernatants containing released 5-125I-chloroform were mixed with scintillation fluid for measuring radioactivity. Aggregation was expressed as the percent of change in light transmission and the 5-125I-chloroform platelet content.

**Calcium Flux Measurements**

Platelets were prepared as described above with slight modifications. Following the resuspension in Tyrode's buffer supplemented with prostacyclin and heparin, platelets were incubated for 30 min at 37 °C with 3 μM Fura 2-acetoxymethylester, washed, and the final platelet concentration was adjusted to 4 × 10⁶/ml in Tyrode's buffer. The basal fluorescence of a 1-ml aliquot of cell suspensions was monitored under stirring with a spectrofluorimeter Jobin Yvon JY 3D (Paris, France) thermostatted at 37 °C. Fluorescence emission and excitation wavelengths were 340 and 510 nm, respectively. Platelets preincubated for 2 min were challenged with cathepsin G, NE, or combinations of both, and the reaction was stopped by addition of 5 μM Wg/ml of cathepsin G (threshold of cathepsin G) preceded by NE, whereas nonfixed cells were used for immunoblot analysis (see below). Next, 10−6 fixed CHO/αthb Cells—Cells were harvested from culture flasks using EDTA buffer and washed once in Iscove's buffer without fetal calf serum and then in Tyrode's buffer. The final pellet was resuspended in this latter buffer such that the final concentration was 10⁷ cells/ml. Then, cells were incubated for 3 min at 37 °C with 400 nM NE or 550 nM cathepsin G under gentle shaking. The reaction was stopped, and part of the cells further fixed with 1% (v/v) formaldehyde, whereas nonfixed cells were used for immunoblot analysis (see below).

**Flow Cytometry Analysis**

**Analysis of Washed Platelets—**Cell samples were treated as for platelet aggregation analysis, except for those used to analyze the binding of PAC-1-Fc, with exogenous fibrinogen omitted as this natural ligand of the activated αthb may competitively inhibit the binding of PAC-1 (31). In any case, once the agonist has been added, the stirring was allowed for only 5 s to homogenize the milieu, then samples were incubated undisturbed for different periods of time at 37 °C to prevent platelet aggregate formation, which would interfere with the flow cytometry analysis. The reaction was stopped by the addition of 5 μM eglin C, an inhibitor of cathepsin G and NE (40) and 2 mM PMSF. Platelets were then immediately fixed with 1% (v/v) formaldehyde for 30 min at room temperature. Following the fixation, all samples were diluted 10-fold in Tyrode's buffer and then incubated for 30 min at 4 °C with saturating concentrations of purified PAC-1 (2 μg/ml), AP-2 (1 μg/ml), PAC-3 (1 μg/ml), AP-5 (diluted 1/1000), or with nonimmune IgG or IgM as control isotypes. Incubations were done in conical bottom plates with 4 × 10⁶ cells/well. After centrifugation of the platelets at 80 × g for 10 min at 4 °C, platelets were washed twice in Tyrode's buffer and incubated for 30 min at 4 °C with the corresponding second fluorescein isothiocyanate-labeled antibody at optimal concentration. Finally, platelets were centrifuged as subsuspended in the same buffer, and stored at 4 °C in the dark until flow cytometric assays were performed within the next 24 h. It is of note that when platelets were to be tested for the expression of the PMI-1 epitope, they were first incubated with the proteinase, then with 5 mM EDTA for 15 min at room temperature to maximally expose the PMI-1 epitope (41), before to be fixed and processed as described above.

**Analysis of CHO/αthb Cells—**Cells were harvested from culture flasks using EDTA buffer and washed once in Iscove's buffer without fetal calf serum and then in Tyrode's buffer. The final pellet was resuspended in this latter buffer such that the final concentration was 10⁷ cells/ml. Then, cells were incubated for 3 min at 37 °C with 400 nM NE or 550 nM cathepsin G under gentle shaking. The reaction was stopped, and part of the cells further fixed with 1% (v/v) formaldehyde, whereas nonfixed cells were used for immunoblot analysis (see below). Next, 10−6 fixed CHO/αthb cells were processed for PAC-1 or AP-2 or control isotype antibodies binding as described for platelets.

In all cases, samples were analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry System, Mountain View, CA). Binding of the different domain-specific anti-αthb antibodies to their epitopes is expressed as the fold increase in median fluorescence intensity over basal values measured on nontreated cells, following subtraction of the background binding measured with the control isotypes.

**Binding of 125I-Labeled Monoclonal Antibodies**

The monoclonal antibodies PMI-1 and AP-5 were purified to homogeneity from ascitic fluids by conventional Protein A- or Protein G-Sepharose chromatography, and labeled with 125I to a specific activity of 4.5-10⁴ becquerels/μg of IgG using the chloramine T procedure (32, 41). Platelets were exposed to NE (400 nM) in an aggregometer cuvette for increasing periods of time (up to 3 min), and the proteinase was blocked by addition of eglin C and PMSF as for flow cytometry analysis. Control platelet suspensions were treated similarly except that NE was absent. Platelets were immediately distributed (final concentration, 2 × 10⁴/ml) in a Tyrode’s medium containing either divalent cations or EDTA (final concentration, 3 mM), and either one of the 125I-labeled antibodies. Incubations were performed at room temperature for 45 min, and platelet-bound antibodies were separated from unbound by layering triplicate 50-μl aliquots of the cell suspensions on 0.5 ml of 20% sucrose made in Tyrode’s medium, and centrifugation for 3 min at 14,000 × g. The supernatant and sucrose were aspirated, and the platelet pellets at the bottom of the tube cut and counted for 125I in a 1282 Compugamma CS counter (LKB Wallac, Turku, Finland). Binding of 125I-AP-5 on NE-treated platelets, which reflected fibrinogen binding to the activated αthbβth integrin (33), was performed in the presence of increasing concentrations of αthb membrane bound to control non-treated platelets was negligible, as previously reported (33), and increased linearly as a constant fraction (0.7%) of the antibody input. This was taken as nonspecific binding. Maximal binding of 125I-AP-5 was measured in the presence of EDTA (33 and found to saturate at 25 μM IgG; this concentration was used throughout all subsequent experiments. Binding of 125I-PMI-1 on NE-treated platelets, which measured the proteolysis of the αthbβth integrin (see “Results”), was performed in the presence of EDTA, to maximize the exposure of the αthb integrin on the platelet surface (41). In preliminary experiments, the nonspecific binding was measured in the presence of a 50-fold excess of unlabeled antibody, and found to represent a constant fraction (0.25%) of the antibody input. Maximal binding of 125I-PMI-1 was measured on control platelets in the presence of EDTA and found to saturate at 25 μM IgG; this concentration was used throughout all subsequent experiments. Isotherm binding of increasing amounts of 125I-labeled antibodies to nontreated or NE-treated platelets for 1 min showed that the KD of each antibody for its epitope was unchanged following proteolysis of αthbβth (data not shown). All data are reported as specific binding, i.e., total binding corrected for the nonspecific as defined above.


**RESULTS**

Characteristics of the Potentiation by NE of Cathepsin G-induced Platelet Activation—As previously established (11, 16), cathepsin G alone added to platelet suspensions at the optimal concentration of 550 nm acts as a strong platelet agonist inducing extensive platelet aggregation (83.5 ± 5.6%, n = 5), accompanied by a marked exocytosis of intracellular granules as judged by the release of 5-[14C]HT from dense granules (76.2 ± 1.6%, n = 5; Fig. 1, panels A and B). For each tested platelet suspension, we determined the threshold of cathepsin G as that resulted in platelet shape change followed by 5–10% of increase in light transmission within 3 min of stirring (Fig. 1, panels A and B). This concentration was always within the range 150 to 180 nm. Under these conditions, exocytosis remained minimal at 3 min, with 3.1 ± 0.8% of 5-[14C]HT release (n = 5; Fig. 1, panel B).

Platelet suspensions stirred for 3 min with 400 nm NE (and up to 800 nm) showed no evidence for aggregate formation (Fig. 1, panel A) and exocytosis of internal granules was barely detectable (0.8 ± 0.3% release of 5-[14C]HT, n = 5), in agreement with previous reports (17–19). By contrast, addition of 400 nm NE 10 s before stimulation of platelets with threshold of cathepsin G resulted in an extensive aggregation, similar to that induced by the optimal concentration of cathepsin G (Fig. 1, panels A and B). The potentiation exerted by NE on threshold of cathepsin G-induced platelet aggregation was already detectable with 100 nm NE, and was maximal in the range 200–800 nm NE (Fig. 1, panel B). Increasing the period of exposure of platelets to NE to 180 s before threshold of cathepsin G had no further effect on the extent of potentiation (not shown and Ref. 18).

Major observations in these experiments were that (i) whereas aggregation induced by 550 nm cathepsin G was associated with an extensive granule exocytosis, the aggregation induced by the combination of 100–800 nm NE with threshold of cathepsin G was accompanied by a limited release of granule contents, varying from 4.7 ± 1.4% to a maximum of 25.9 ± 0.9% secretion of 5-[14C]HT (n = 5; Fig. 1, panel B); and (ii) the potentiating activity of NE on threshold of cathepsin G-induced platelet aggregation was strictly dependent on the presence of exogenous fibrinogen (Fig. 1, panels A and C).

**Activation of the Platelet Fibrinogen Receptor by NE and Cathepsin G**—Previous reports have shown that platelet exposure to various serine proteinases, including elastases, induces expression of fibrinogen binding sites (26, 27). This, together with the requirement for exogenous fibrinogen for the potentiation of platelet aggregation (the present work) led us to consider that the synergy resulting from the combination of NE and threshold of cathepsin G could be exerted at the level of the αIIbβ3 integrin, the platelet fibrinogen receptor. We first evaluated whether NE could modify itself the surface expression and the biological activity of αIIbβ3 on platelets by using flow cytometry analysis with a panel of monoclonal antibodies specific for distinct conformations of this integrin (see Table 1). These included AP-2, an αIIbβ3 complex-specific antibody reacting with both the resting and active forms of the receptor (32), PAC-1, which binds at one fibrinogen-binding site on αIIbβ3, and only recognizes the active conformation of the receptor (31), and AP-5, an anti-ligand-induced binding site (LIBS) which specifically reveals the active and fibrinogen-occupied integrin (33). As mentioned under “Materials and Methods,” when platelets were to be tested with PAC-1, prior incubation with NE was without exogenous fibrinogen, while analysis of the binding of AP-5 was necessarily performed on platelets incubated in the presence of fibrinogen.

As illustrated in Fig. 2, exposure of platelet suspensions to...
400 nM NE at 37 °C, a concentration which triggers a maximal potentiation of threshold of cathepsin G-induced platelet aggregation, resulted in a rapid transition of the \( \alpha_{IIb}\beta_3 \) conformation reflecting activation of the receptor and binding of its ligand. Indeed, a time-dependent increase in binding of PAC-1 occurred within 10 s of exposure to NE, and reached a plateau after 1 min (panel A). A similar increase in the binding of AP-5 was observed, with a plateau after 2 min of exposure to NE (panel B). At 3 min, binding of PAC-1 and AP-5 on NE-treated platelets was increased about 2.3-fold \((n = 8, p < 0.001)\) and 3.4-fold \((n = 4, p < 0.05)\), respectively, compared with nontreated platelets. However, binding of these antibodies on NE-treated platelets was approximately 3.5-fold lower than that measured on platelets optimally stimulated with 550 nM cathepsin G (compare panels A and C, and B and D). Such a difference can be largely explained by the ability of a high concentration of cathepsin G to induce platelet shape change and extensive exocytosis of \( \alpha \)-granules (43), thus allowing the translocation of the internal fraction of \( \alpha_{IIb}\beta_3 \) complexes to the plasma membrane (25). Indeed, binding of AP-2 to platelets activated with 550 nM cathepsin G for 3 min was increased by 92.7 \( \pm \) 16.8% when compared with nontreated platelets \((n = 7, p < 0.001; \text{see Fig. 3, panel B})\), an increase similar to that measured on platelets activated with 0.5 IU/ml thrombin.
Involvement of the activation of α_{IIIb}β_{3} by NE in the Potentiation of Threshold of Cathepsin G-induced Platelet Aggregation—Considering that NE is able to up-regulate the biological activity of the plasma membrane α_{IIIb}β_{3} integrin, we assumed that the potentiation by NE of platelet aggregation induced by threshold of cathepsin G resulted from an increased capacity of the platelet surface to bind fibrinogen. To examine this hypothesis, platelet suspensions were challenged for 3 min with 400 nM NE, threshold of cathepsin G, or a combination of both proteinases, before being processed for analysis of AP-2 and PAC-1 binding by flow cytometry, the latter antibody being taken as a fibrinogen-like probe. Thus, combination of the two proteinases increased the binding of PAC-1 ~5.5-fold, while activation of platelets with 550 nM cathepsin G increased PAC-1 binding ~7.5-fold, these values being not statistically different (p > 0.05, n = 3). By contrast, the increase in PAC-1 binding induced at 3 min by threshold of cathepsin G alone was ~3-fold above the background binding measured for nontreated platelets, a value similar to that measured for NE-treated platelets in this series of experiments (p > 0.05; Fig. 3, panel A). Of note is that PAC-1 binding to platelets activated with either threshold of cathepsin G alone or with the combination of NE and threshold of cathepsin G was significantly different (p < 0.05, n = 3). When platelet suspensions were similarly treated in the presence of exogenous fibrinogen, then evaluated for the binding of AP-5 as a marker of ligand-occupied α_{IIIb}β_{3} similar profiles were obtained (not illustrated). Strikingly, the strong potentiating effect of NE on threshold of cathepsin G-induced platelet aggregation occurred despite a limited expression of the internal α_{IIIb}β_{3} fraction at the plasma membrane, as measured by the binding of AP-2 which was identical to that initiated by threshold of cathepsin G alone (~1.3-fold increase over basal value under both conditions, p > 0.05; Fig. 3, panel B).

Role of the Platelet Intracellular Signaling in the Potentiation Induced by the Combination of NE and Cathepsin G—The enhanced exposure of fibrinogen-binding sites induced by NE could be potentially explained by the ability of this proteinase to enhance intracellular signals by which cathepsin G normally initiates platelet activation and thus up-regulates the activity of α_{IIIb}β_{3}. It has been demonstrated (14–16) that the interrelated elements of the PLC-Ca^{2+}-PKC pathway act in concert to mediate such a cell response. Thus, as indicated in Fig. 4, an optimal concentration of cathepsin G (550 nM) triggered extensive activation of the PLC and PKC pathways, as measured through PtdOH accumulation (panel A) and phosphorylation of p47, a 47-kDa protein (P47) which is the main substrate for PKC in platelets (44) (panel B). As expected, activation of PLC and PKC was accompanied by a massive increase in cytosolic Ca^{2+} (panel C). By contrast, activation of platelets with threshold of cathepsin G resulted in a detectable but limited metabolic activation compared with 550 nM cathepsin G. On the other hand, exposure of platelets to NE alone at concentrations up to 800 nM failed to initiate PLC or PKC activities or intracellular Ca^{2+} movements. Finally, and more importantly, stimulation of platelets with the combination of 400 nM NE and threshold of cathepsin G resulted in an activation which was not different with that produced by threshold of cathepsin G alone (values of fold-increase over basal signals were 2.25 ± 0.25 and 2.63 ± 0.14 (n = 4) for PtdOH accumulation, respectively, and 1.26 ± 0.05 and 1.24 ± 0.02 (n = 4) for P47 phosphorylation, respectively). Furthermore, we determined that the activation of α_{IIIb}β_{3} by NE was not blocked by substances known to prevent platelet activation. Table II shows that pretreatment of platelets with GF 109203X, a specific PKC inhibitor (45), or with PGL, a potent activator of platelet adenylate cyclase and inhibitor of α_{IIIb}β_{3} metabolic activation (46), had no

**Fig. 2.** Activation of the platelet fibrinogen receptor by NE and cathepsin G. Unstimulated platelets were preincubated in the absence (panels A and C) or presence (panels B and D) of exogenous fibrinogen (0.7 mg/ml) to analyze PAC-1 or AP-5 antibodies binding, respectively. Reactions were initiated by adding 400 nM NE (panels A and B) or 550 nM cathepsin G (panels C and D) and stopped at different times with 5 μM eglin C and 2 mM PMSF. Binding of PAC-1 and AP-5 to platelets were then measured by flow cytometry and are expressed as the fold-increase in median fluorescence intensity over basal values measured on nontreated platelets. Results are means ± S.E. of four to eight experiments conducted with cells from different donors.

(98.9 ± 9.2%, n = 8, p < 0.001). By contrast, the increase in AP-2 binding to platelets exposed to 400 nM NE was only 9.6 ± 5.0% and remained nonsignificant (n = 8, p > 0.05; see Fig. 3, panel B).

**Fig. 3.** Involvement of the activation of α_{IIIb}β_{3} by NE in the potentiation of threshold of cathepsin G-induced platelet activation. Unstimulated platelets were incubated in the absence of exogenous fibrinogen. Reactions were initiated by adding 400 nM NE, threshold of cathepsin G (150–180 nM) alone or in combination with NE, or 550 nM cathepsin G. Reactions were followed for 3 min and stopped with eglin C and PMSF. Bindings of PAC-1 (panel A) and AP-2 (panel B) measured by flow cytometry are expressed as the fold increase in median fluorescence intensity over basal values measured on nontreated platelets. Results are means ± S.E. of three experiments conducted with cells from different donors. thCat.G, threshold of concentration of cathepsin G.
**α<sub>IIb</sub>β<sub>3</sub> and Platelet Activation by Elastase and Cathepsin G**

Inhibitory effect on the capacity of 400 nM NE to activate α<sub>IIb</sub>β<sub>3</sub>, as evaluated by the binding of PAC-1. By contrast, and as expected, both GF 109203X and PGLI were potent inhibitors of the expression of activated α<sub>IIb</sub>β<sub>3</sub> on the surface of platelets stimulated with 0.5 IU/ml thrombin (45, 46). Altogether, these data clearly indicate that the potentiating effect of NE on threshold of cathepsin G-induced platelet aggregation is not related to an increased intracellular signaling involving the PLC, PKC, and Ca<sup>2+</sup> components.

**Effects of NE on the Structure of α<sub>IIb</sub>β<sub>3</sub>—** A major feature in our study was that PMSF-inactivated NE was totally unable to either up-regulate the activity of α<sub>IIb</sub>β<sub>3</sub> as measured by the binding of PAC-1 (2.3 ± 0.2- versus 1.1 ± 0.1-fold increase (n = 3) over basal values with intact and PMSF-treated NE, respectively), or to potentiate cathepsin G-induced platelet aggregation (not shown and Ref. 19), indicating that the proteolytic activity of NE is required for both processes. This, together with the known susceptibility of α<sub>IIb</sub>β<sub>3</sub> to proteolysis by serine proteinases (26–30) and the absence of intracellular metabolic activation by NE (see above), prompted us to examine whether the integrin structure was affected by this proteinase under our experimental conditions.

**Panel A** in Fig. 5 illustrates the analysis of the β<sub>3</sub> and α<sub>IIb</sub> subunits separated on 7–12% gradient acrylamide gels following reduction of intra- or interchain disulfide bonds, and probed with polyclonal rabbit antisera raised against each of the whole subunit. When compared with nontreated control platelets (lane 1), platelets exposed to 400 nM NE for 3 min (lane 2) showed no proteolytic modification of the β<sub>3</sub> subunit (M<sub>r</sub> ~ 118,000), whose mobility and intensity remained unchanged. Only longer exposure (15 min) to 400 nM NE, or to higher concentrations of proteinase (1.2 μM) resulted in the appearance of a minor membrane-associated fragment of β<sub>3</sub> with M<sub>r</sub> ~ 66,000 (not shown and Ref. 27). Under the conditions of electrophoresis used in this experiment, the α<sub>IIb</sub> subunit could be clearly resolved into its two heavy and light polypeptide chains (α<sub>IIbH</sub>, M<sub>r</sub> ~ 126,000, and α<sub>IIbL</sub>, M<sub>r</sub> ~ 25,000). α<sub>IIbH</sub> appeared to be unchanged for both its mobility and intensity in NE-treated platelets. By contrast, the α<sub>IIbL</sub> subunit migrated as a broader band in platelets exposed to NE (lane 2), as compared with nontreated platelets (lane 1), with a component (indicated by the open arrowhead in Fig. 5, panel A) running slightly ahead of the intact α<sub>IIbH</sub>.

More detailed immunoblot analysis of the α<sub>IIbH</sub> subunit was performed following reduced SDS-PAGE on highly resolutive 5% acrylamide gels, using a panel of domain-specific α<sub>IIb</sub> antibodies (see Table I). As illustrated in panel B of Fig. 5, the anti-α<sub>IIb</sub> polyclonal antiserum clearly identified two molecular species in NE-treated platelets (lane 2), showing approximately equal intensity, one corresponding to the intact α<sub>IIbH</sub> (M<sub>r</sub> ~ 128,000) as seen in control untreated platelets (lane 1), and the second to a membrane-bound proteolytic fragment with M<sub>r</sub> ~ 123,000, designated α<sub>IIbH</sub>. On several platelet samples exposed to NE under identical conditions, the mean M<sub>r</sub> difference between α<sub>IIbH</sub> and α<sub>IIbH</sub> was 6,470 ± 290 (n = 15). Considering that α<sub>IIbH</sub> is entirely extracellular, such a limited proteolysis must have occurred at one or both extremities of the polypeptide chain. The polyclonal rabbit antiserum IIb-10, recognizing the α<sub>IIbH</sub> amino-terminal Leu<sup>1</sup>-Pro<sup>14</sup> sequence (30), reacted equally with the intact α<sub>IIbH</sub> and the α<sub>IIbH</sub> fragment. By contrast, the murine monoclonal antibody PMI-1, which recognizes the α<sub>IIbL</sub> carboxyl-terminal Pro<sup>404</sup>-Arg<sup>406</sup> sequence (34), was reactive with the residual intact α<sub>IIbH</sub> in NE-treated platelets, but totally unreactive with α<sub>IIbH</sub> (Fig. 5, panel B). To ascertain that proteolysis was limited to α<sub>IIbH</sub>, similar experiments were performed on unreduced samples (Fig. 5, panel C). Here, the anti-α<sub>IIb</sub> antiserum identified the native α<sub>IIb</sub> subunit (i.e. disulfide-linked α<sub>IIbH</sub> and α<sub>IIbL</sub>) in nontreated samples with M<sub>r</sub> ~ 143,000 (lane 1). With NE-treated platelets (lane 2), a second component could be distinguished slightly ahead of intact α<sub>IIb</sub>. This component, designated α<sub>IIbH</sub>, had M<sub>r</sub> ~ 137,000. The α<sub>IIb</sub> light chain within the α<sub>IIbH</sub> membrane-bound proteolytic species was shown to have an intact amino terminus by the normal reactivity of α<sub>IIbH</sub> with the anti-V41 polyclonal antiserum, which recognizes the amino-terminal Gln<sup>260</sup>-Arg<sup>271</sup> sequence of α<sub>IIbH</sub> (34).

Similar immunoblot analysis was further performed on SDS lysates of platelets treated for 3 min with the combination of 400 nM NE and threshold of cathepsin G (lanes 3 in Fig. 5). Results were strictly identical to those obtained with platelets treated with 400 nM NE alone. In addition, we previously demonstrated that high concentrations of cathepsin G alone have no proteolytic effect on the α<sub>IIb</sub>β<sub>3</sub> integrin (47). Taken together, these data indicate that under optimal conditions of potentiation by NE of threshold of cathepsin G-initiated aggregation, NE specifically proteolyses a short domain located at the carboxyl terminus of the α<sub>IIbN</sub> polypeptide chain.

Since the above data pointed to the existence of a specific and previously unreported modification of α<sub>IIb</sub>β<sub>3</sub> by NE, we sought to confirm that it occurred to a significant extent at the surface...
of platelets. Platelet suspensions were thus exposed to 400 nM NE for increasing periods of time (up to 3 min) and analyzed by flow cytometry to quantitate the binding of the monoclonal antibody PMI-1 (this being taken as a marker of proteolysis at the αIIbβ3 carboxyl terminus) since the PMI-1 epitope should be lost upon exposure to NE. Results indicated a near complete disappearance of the PMI-1 epitope on NE-treated platelets, with a binding after 3 min of proteolysis decreased by 93.4% (n = 5) compared with the initial value measured on untreated platelets (data not illustrated).

Localization of the Cleavage Site(s) for NE within the αIIbβ3 Carboxyl Terminus—Considering the domain of αIIbβ3 proteolysis by NE (see above), the relative M₉ difference measured between αIIbH₃ and αIIbH₅ (≈6,500), and the fact that in resting platelets, the epitope recognized by PMI-1 is largely cryptic (41), the site(s) of cleavage by NE were searched by MALDI-TOF mass spectrometry on a peptide corresponding to the sequence Phe827-Leu841 of αIIbH. Indeed, this sequence maps from the carboxyl-terminal side of Cys826, which is involved in the linkage of αIIbH to αIIbL (20), to the amino-terminal side of the PMI-1 epitope (34) (Fig. 6, panel B). The lower tracing in panel A of Fig. 6 shows a mass spectrum which corresponds to the initial undigested peptide 827–841, with a mass of 1708.0 Da. Upon a 5-min incubation of this peptide with 400 nM NE (upper tracing in panel A of Fig. 6), a single new peptide was generated with a mass of 1236.8 Da, corresponding to the sequence Phe827-Val837. This indicates a proteolysis of the initial peptide at a unique bond, between Val837 and Asp838. Of
note is that the cleavage was detectable as soon as 30 s and was complete at 10 min, without detection of other proteolytic products (not shown). The shorter tetrapeptide fragment Asp-Phe-Leu841, corresponding to the amino acid sequence Phe827 to Leu841 of the αIIbβ3 subunit, heavy chain. Panel A, MALDI-TOP mass spectra of the peptide 827-841, corresponding to the amino acid sequence Phe827 to Leu841 of the αIIbβ3 subunit. Lower and upper tracings are mass spectra of the untreated peptide and of peptides obtained after a 5-min digestion by NE at 37 °C, pH 7.4, respectively (m/z, observed mass/charge). Tracings are representative of two distinct experiments performed in duplicate. Panel B, location of the deduced cleavage site by NE (indicated by a vertical arrow) in the αIIbβ3 carboxyl terminus. Epitopes of the PM-1 and anti-V41 antibodies within the αIIbβ3 subunit are also indicated. The shaded circle at Ser847 represents an O-linked oligosaccharide (20).

**Fig. 6.** Localization of the cleavage site for NE within the carboxyl terminus of the αIIbβ3 subunit heavy chain.

**Panel A:** MALDI-TOP mass spectra of the peptide 827-841, corresponding to the amino acid sequence Phe827 to Leu841 of the αIIbβ3 subunit. Lower and upper tracings are mass spectra of the untreated peptide and of peptides obtained after a 5-min digestion by NE at 37 °C, pH 7.4, respectively (m/z, observed mass/charge). Tracings are representative of two distinct experiments performed in duplicate. Panel B, location of the deduced cleavage site by NE (indicated by a vertical arrow) in the αIIbβ3 carboxyl terminus. Epitopes of the PM-1 and anti-V41 antibodies within the αIIbβ3 subunit are also indicated. The shaded circle at Ser847 represents an O-linked oligosaccharide (20).

**Relationship between Cleavage of the αIIb Subunit Heavy Chain by NE and Activation of αIIbβ3—**When bindings of both PMI-1 and AP-5 were examined by flow cytometry on the same platelet samples treated by NE, a relationship was observed between the time course of proteolysis of the αIIbβ3 subunit (i.e. the decreasing binding of PMI-1) and that of the ligand binding capacity of αIIbβ3 (i.e. the increasing binding of AP-5) (data not shown). To further support this inference and to exclude an activation of the integrin resulting from NE acting on another membrane structure inducing signaling which may feed-back to αIIbβ3, two series of experiments were carried out. First, platelets were depleted in cytosolic ATP by incubation for 15 min with 50 mM 2-deoxy-d-glucose, 0.05% NaN3, and 10 mM glucono-δ-lactone to inhibit glycolysis, oxidative phosphorylations, and glycogen phosphorylase, respectively (48). These platelets were treated or not with 400 nM NE or 550 nM cathepsin G for 3 min, and PAC-1 binding was examined by flow cytometry as an index of αIIbβ3 activation. Results showed that while NE-induced activation of the fibrinogen receptor remained unchanged (p > 0.05, n = 3), that produced through intracellular pathways by cathepsin G (Fig. 4) was inhibited by 73.8 ± 1.0% (p < 0.001, n = 3). Second, CHO cells expressing human αIIbβ3 were used as it is known that these cells do not support agonist-induced metabolic activation of this integrin (49). Fig. 7 indicates that treatment of CHOαIIbβ3 cells with NE resulted in a 4.2 ± 0.7-fold increase over basal value for PAC-1 binding (n = 2). This was specific to NE since incubation of these cells with 550 nM cathepsin G did not trigger any increase in PAC-1 binding. Moreover, this process did not result from an increase of αIIbβ3 molecules expression at the surface of CHOαIIbβ3 cells as values for AP-2 binding were unchanged compared with nontreated cells. Importantly, in both cases (i.e. platelets pretreated with metabolic inhibitors and CHOαIIbβ3 cells), activation of αIIbβ3 by NE was accompanied by an extensive proteolysis of αIIbβ3 (Fig. 7).

We thus further examined whether cleavage of the αIIbβ3 subunit by NE quantitatively correlates with the activation of αIIbβ3. Direct binding of 125I-AP-5 and 125I-PMI-1 antibodies allowed quantitative measurements of the number of NE-activated and occupied αIIbβ3 molecules in relation to the number of proteolyzed molecules, respectively. Panel A in Fig. 8 shows...
that the maximal number of binding sites for $^{125}$I-PMI-1 on control nontreated platelets (27,270 ± 5,360 molecules/platelet, n = 3) progressively decreased upon exposure of cells to NE, to be reduced at 3 min by 96 ± 3%. Panel B demonstrates that, on a time course basis, the proteolysis of the carboxyl-terminal domain of $\alpha_{IIb}$ correlates linearly with the increased capacity of $\alpha_{IIb}\beta_3$ to bind exogenous fibrinogen ($r^2 = 0.902, p \leq 0.005$). In this series of experiments, the maximal binding of $^{125}$I-AP-5 (measured in the presence of EDTA) on nontreated as well as NE-treated platelets amounted to 18,250 ± 770 molecules/platelet (n = 3), whereas the specific binding measured in the presence of divalent cations and fibrinogen on platelets treated with NE for 3 min amounted to 4,270 ± 760 molecules/platelet, i.e. 23 ± 3.6% of the maximal AP-5 binding capacity of platelets. These data suggest that not all proteolyzed $\alpha_{IIb}\beta_3$ molecules acquire the capacity to bind a ligand, and that the stoichiometry is about one active integrin over four proteolyzed.

**DISCUSSION**

The aim of the present investigation was to characterize the molecular mechanism underlying the synergistic activation of platelets by the neutrophil-derived proteinases elastase and cathepsin G. The major findings are as follows: (i) NE does not activate the platelet intracellular signaling but specifically cleaves the $\alpha_{IIb}$ subunit heavy chain of the $\alpha_{IIb}\beta_3$ integrin, likely between Val$^{837}$ and Asp$^{838}$; (ii) this proteolysis correlates with an up-regulation of the fibrinogen receptor function of this integrin; and (iii) this particular activation of $\alpha_{IIb}\beta_3$ by NE is relevant for the potentiation of platelet aggregation initiated by low concentrations of cathepsin G.

The $\alpha_{IIb}\beta_3$ integrin has been shown to undergo variations in affinity for fibrinogen that reflect conformational changes within the $\alpha_{IIb}$ and $\beta_3$ subunits (20–22). Since cathepsin G stimulates platelets as potently as does thrombin and through a common signaling pathway (14–16, 50), it may be speculated that the mechanism which regulates the fibrinogen-binding function is similar for both proteinases. In thrombin-activated platelets, an intracellular signal transduction pathway, including heterotrimeric GTP-binding proteins, PLC, PKC, PI 3-kinase, low molecular weight GTP-binding proteins and the cytoskeleton, affects the cytoplasmic domains of $\alpha_{IIb}\beta_3$ and influence the conformation of the extracellular domains (inside-out signaling) (22, 51). Hence, cathepsin G-induced activation of the $\alpha_{IIb}\beta_3$ integrin (the present study and Ref. 43 and 50) is likely controlled by this complex network of intracellular signaling reactions. In addition to that exposed on the cell surface, $\alpha_{IIb}\beta_3$ has been identified in an internal membrane compartment made of the surface-connected canalicular system and the α-granules (24). Compared with resting platelets, the density of $\alpha_{IIb}\beta_3$ on the platelet plasma membrane increases after strong stimulation, due to the mobilization of these intracellular stores (25). This is confirmed in the present work for a maximal concentration of cathepsin G (550 nM) using AP-2, a monoclonal antibody that recognizes a complex-dependent determinant on $\alpha_{IIb}\beta_3$, whose binding doubles under these conditions, in agreement with previous findings (50).

Other serine proteinases have been shown to induce the activation of $\alpha_{IIb}\beta_3$, but through a nonmetabolic pathway. Thus, treatment of platelets with chymotrypsin (28–30) or elastases (26, 27) results in a proteolytic-dependent exposure of fibrinogen-binding sites at the platelet surface. In accordance with these studies, measurements of the binding of PAC-1 and AP-5 antibodies allowed us to demonstrate that NE rapidly increases the high affinity and ligand-occupied conformers of native $\alpha_{IIb}\beta_3$. Unlike cathepsin G or thrombin, this was not accompanied by an increase of the number of $\alpha_{IIb}\beta_3$ expressed on the platelet surface. Another difference with cathepsin G or thrombin is that the up-regulation of $\alpha_{IIb}\beta_3$ by NE occurs even in the presence of the potent inhibitor of platelet activation PGI2 or after the metabolic pool of ATP has been depleted. A similar stimulation of fibrinogen binding at the platelet surface, independent of PGI2-inhibitable pathways, has been observed following incubation of platelets with Fab fragments of certain anti-LIBS antibodies (52). It was hypothesized that these activating antibodies shift a conformational balance to...
favor or stabilize the high affinity of αIIbβ3 for fibrinogen. With regard to NE, its potential binding to the integrin might also displace a structural equilibrium as do anti-LIBS antibodies. However, our data rather indicate that the conformational shift which uncovers binding site(s) for fibrinogen is associated with a proteolytic processing. Indeed, the blockade of NE catalytic site by PMSF suppressed the activation shift which uncovers binding site(s) for fibrinogen is associated with a proteolytic processing. Therefore, kinetics of this cleavage closely correlated with that of the expression of the ligand binding activity. It is of note, however, that these two events occur in a stoichiometry of about four αIIbβ3 molecules proteolyzed for one molecule activated. Taken together, these data mean that NE cleaves almost all the αIIbβ3 complexes expressed at the platelet plasma membrane, but only a fraction (∼25%) of them shift from an inactive to an active conformer. This observation is actually consistent with studies which have brought evidence for the existence of distinct subpopulations of αIIbβ3 at the platelet surface, showing distinct conformations and/or susceptibility to acquire an activated state (52–54). Interestingly, this functional heterogeneity appears intrinsic to the αIIbβ3 molecules. Thus, after purification of the total platelet integrin, at least two conformers can be identified, one inactive but still activable, and one “naturally” active following solubilization (∼20% of the total, based on data reported in Ref. 54). The mechanism through which a fraction of the platelet αIIbβ3 molecules resists the activation shift remains to be determined. Finally, that NE activates αIIbβ3 directly through proteolysis was confirmed using CHO cells transfected with human αIIbβ3, knowing that the integrin expressed in these cells is unable to respond to metabolic activation (49). All these data concur to exclude that NE acts on another membrane protein which may secondarily stimulate αIIbβ3.

MALDI-TOF mass spectrometry performed on the synthetic peptide corresponding to the sequence Phe827-Leu841 of αIIbH gave an insight into the site of NE-induced proteolysis by indicating a cleavage located between Val837 and Asp838. This finding is in agreement with the primary specificity of NE for valine residues (55). As a result of this cleavage by NE within the αIIbH carboxyl terminus, a peptide of 19 amino acids, i.e. Asp838-Arg856, including an O-glycosylated serine residue at position 847 (20) (see Fig. 6), is expected to be released. The difference in Mᵣ found in SDS-PAGE between αIIbH and its membrane-bound proteolytic derivative (∼6,500) is in fair agreement with the presumed mass of such a glycopeptide. A mechanism by which this restricted cleavage can modulate global conformational changes within the whole αIIbβ3 integrin can be hypothesized on the basis of different structural models of this integrin (see Fig. 9). From the model proposed by Honda et al. (33), it can be suggested that the αIIbH domain proteolyzed by NE is located in a structurally constrained cluster of cryptic LIBS epitopes including PMI-1 (αIIbH 542–856), AP-5 (β₃ 1–6), LIBS-2 (β₃ 602–690), and an undefined region in β₃ interacting with the PMI-2 antibody. On the other hand, studies aimed to localize the αIIb sequences involved in the intrasubunit contacts have suggested that αIIbH would be folded on itself, notably through interactions between its amino-terminal and carboxyl-terminal domains (56). Therefore, it may be speculated that through limited proteolysis of the carboxyl-terminal end of the αIIbH subunit, NE removes a constraint in a confined but particularly sensitive region. The generated conformational change may then propagate over the whole αIIbβ3 complex (57), converting a subpopulation of the integrin molecules from a resting to an active fibrinogen receptor.

While the PLC/PKC pathway controls the synergism resulting from combination of low concentrations of platelet agonists such as thrombin and epinephrine (58), it does not account for the enhanced activation of αIIbβ3 and platelet aggregation induced by the combination of NE and cathepsin G (see Fig. 4). As one consequence, it can be ruled out that this process occurs through an increase by NE of the catalytic activity of cathepsin G, or an increase of cathepsin G receptor expression and/or affinity, which would have resulted in an increase of intracellular signaling messengers. In fact, on the basis of the foregoing data, and notably those showing the activation by NE of surface-expressed αIIbβ3, a hypothetical mechanism may be pro-

![Schematic model of the activation by NE of the αIIbβ3 integrin through proteolysis of the αIIbH subunit.](Image)
posed to construe how NE enhances the platelet aggregation initiated by low concentrations of cathepsin G. During this process, cathepsin G would initiate different intracellular transduction signals, including the PLC-Ca²⁺-PKC pathway, with as one result, a basal metabolic activation of αIIbβ3, however, insufficient to promote stable aggregate formation. In parallel, NE would trigger a proteolysis of the carboxyl termini of the αIIbβ3 subunit heavy chain with the subsequent partial reorientation of the extracellular domains within the αIIb and β3 subunits allowing increased binding of fibrinogen. As more fibrinogen binds and cross-links adjacent platelets, post-occlusion outside-in signaling events through αIIbβ3 may amplify cell activation (51), and finally maximize the recruitment of platelets to aggregates similar to those produced by high concentrations of strong platelet agonists acting through a solely intracellular signaling pathway. Consistent with this proposal, it is of interest to note that the potentiating effect of NE is not restricted to cathepsin G, as NE can also enhance platelet aggregation initiated by low concentrations of collagen or the thromboxane A₂ stable analog U46619 (not shown and Ref. 19).

Numerous studies suggest that platelet activation in vivo could be attributed to the combined action of pairs (or more) of agonists (58, 59). The potentiation by NE of cathepsin G-induced platelet activation reported in the present work might be relevant in physiopathological situations in which both platelets and neutrophils can locally accumulate and physically interact such at sites of inflammatory or thrombotic lesions (4–6). In connection with these studies, it is tempting to speculate that proteinase-induced enhancement of adhesive receptor function could be a wide and physiologically relevant process. Indeed, like the proteolysis-dependent activation of αIIbβ3 by NE described here, a recent study provided evidence that serine proteinases proteolytically enhance cell adhesion mediated by the integrin αIIbβ3 (60).

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REFERENCES

1. Cerletti, C., Evangelista, V., Molino M., and de Gaetano, G. (1995) Thromb. Haemostasis 74, 218–223
2. Marcus, A. J., Saffer, L. B., Broekman, M. J., Islam, N., Fliesbach, J. H., Hajar, K. A., Kaminski, W. E., Jendrachek, E., Silverstein, R. L., and von Schacky, C. (1995) Thromb. Haemostasis 74, 213–217
3. Del Maschio, A., Dejana, E., and Bazzoni, G. (1993) Ann. Hematol. 67, 23–31
4. Issekutz, A. C., Ripley, M., and Jackson, J. R. (1983) J. Cardiovasc. Pharmacol. 5, 99–103
5. Bednar, M., Smith, B., Pinto, A., and Mullane, K. M. (1985) Circulation 71, 424–431
6. Ishak, K. G., and Scully, M. (1982) Hepatology 2, 263–266
7. Borkowski, K., Kaczanowska, J., Karpowicz, M., Stachurska, J., and Kopec, M. (1983) Thromb. Haemostasis 50, 768–772
8. Nakajima, K., Powers, J. C., Ashe, B. M., and Zimmerman, M. (1979) Am. J. Pathol. 94, H870–H879
9. Renesto, P., and Chignard, M. (1991) Lab. Invest. 64, 69–76
10. Kornecki, E., Melchior, C., Guinet, J. M., Michels, S., Gouon, V., and Bron, N. (1996) Cell Adhes. Com. 4, 25–39
11. Konttinen, U. T. (1970) Nature 227, 580–585
12. Jolles, J., Zwiers, H., Dekker, A., Wirtz, K. W. A., and Gispen, W. H. (1981) Biochem. J. 194, 283–291
13. Frelinger, A. L., III, Lam, S. C.-T., Plow, E. F., Smith, M. A., Loftus, J. C., and Hawiger, J. (1982) J. Biol. Chem. 257, 14606–14609
14. Molino, M., Di Lallo, M., de Gaetano, G., and Cerletti, C. (1992) Biochem. J. 288, 741–745
15. Selak, M. A. (1990) Platelets 4, 85–89
16. Si-Tahar, M., Renesto, P., Falet, H., Rendu, F., and Chignard, M. (1996) Biochem. J. 313, 401–408
17. Bykowska, K., Kaczanowska, J., Karpowicz, M., Stachurska, J., and Kopec, M. (1983) Thromb. Haemostasis 50, 768–772
18. Selak, M. A. (1992) Thromb. Haemostasis 68, 570–576
19. Renesto, P., and Chignard, M. (1993) Blood 82, 139–144
20. Calvete, J. J. (1994) Thromb. Haemostasis 72, 1–15
21. Ginsberg, M. H., Xu, D., O’Toole, T. E., and Lofus, J. C. (1995) Thromb. Haemostasis 74, 352–359
22. Steuver, I., and O’Toole, T. E. (1990) Stem Cells 9, 250–262
23. Hynes, R. O. (1992) Cell 69, 11–25
24. Wencel-Drape, J. D., Plow E. F., Kunicki, T. J., Woods, V. L., Keller, D. M., and Ginsberg, M. H. (1986) Am. J. Pathol. 124, 324–334
25. Nüya, K., Hudson, E., Bader, R., Byers-Ward, V., Koniz, J. A., Plow, E. F., and Rudiger, Z. M. (1987) Blood 70, 475–483
26. Kornecki, E., Ehrlich, Y. H., De Mars, D. M., and Lenox, R. H. (1986) J. Clin. Invest. 77, 756–756
27. Konttinen, U. T., and platelet activation by elastase and cathepsin G 41. Frelinger, A. L., III, Lam, S. C.-T., Plow, E. F., Smith, M. A., Loftus, J. C., and Hawiger, J. (1982) J. Biol. Chem. 257, 14606–14609