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# Contribution of activated beta3 integrin in the PDI release from endothelial cells

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## 1. ABSTRACT

Protein disulfide isomerase (PDI) is an abundant reticulum endoplasmic protein but also acts as an important functional regulator of some extracellular surface proteins. Recent studies suggest that PDI plays a role in integrin activation and thrombus formation. The aim of this study was to examine whether activation of integrin is the first stage leading to release of PDI from the subcellular compartments of endothelial cells to extracellular space. Our results show that endothelial cells which adhere to fibronectin or fibrinogen release significantly more PDI than those which adhere to poly-L-lysine. Cells treated with RGD peptide, Src and FAK kinase inhibitors and anti alphaVbeta3 antibody display lower PDI secretion. The destruction of the actin cytoskeleton of endothelial cells by cytochalasin D inhibits PDI release. When the endothelial cells adhere to fibrinogen or fibronectin, PDI and alphaVbeta3 gain free thiol groups. Our data suggest that upon activation of integrins, PDI is released from endothelial cells and forms a disulfide bond complex with alphaVbeta3 integrin.

## 2. INTRODUCTION

PDI, a member of the thiol-disulfide oxidoreductase family, displays thiol isomerase, oxidase and reductase activity. PDI is found on the surface of several types of cells, including endothelial cells, hepatocytes, pancreatic cells, neutrophils and cancer cells (1). It has also been found on the surface of platelets, where it plays an important role in platelet

aggregation and secretion (2). Extracellular PDI is known to mediate in integrin activation (3,4).

Integrins are the major cellular receptors involved in signal transduction associated with adhesion, migration, proliferation, cell differentiation and apoptosis. They integrate intracellular and extracellular environments using bidirectional signaling. Integrin activation is accompanied by a number of conformational rearrangements, resulting in changes in affinity and avidity (5). The binding of physiological ligands to integrins is triggered allosterically by "inside-out" activation signals propagated across the plasma membrane to induce ligand occupancy. Ligand-occupied integrins initiate signals that travel "outside-in" to modify cell behavior (6). Integrins contain several highly conserved cysteine residues. Some of the cysteines have disulfide bonds and some contain free thiols (7). Conformational changes induced by ligand interactions with integrin lead to the exchange of disulfide bonds within the integrin molecule, which stabilizes the altered conformation, thus enabling sustained binding. The formation and rearrangement of disulfide bonds is modulated by protein disulfide isomerase (PDI) (8,9).

PDI is present on surface of endothelium where it forms a complex with alphaVbeta3 integrin. Endothelial PDI has been identified in several subcellular compartments, where it plays a critical role in protein folding and in disulfide bond formation in the nascent protein. In endothelial cells, PDI partially colocalizes with GRO-alpha in small granules (3). Extracellular PDI is known to be present during thrombus formation following injury of the vascular wall (3). PDI is secreted by endothelial cells (3,10) but the physiological mechanism of secretion of PDI from endothelial cells is not well defined.

Recent observations suggest that PDI is released from subcellular compartments to the cytosol and is translocated towards the cell membrane where it forms a complex with beta-actin in adhering cells (11). It is possible that PDI is recruited to the adhesion area by active form of integrins during cell adhesion.

The present study examines whether activation of integrins by adhesion plays a crucial role in the initiation of signals which may lead to the release of PDI from the subcellular compartments of endothelial cells to the extracellular space.

## 3. MATERIALS AND METHODS

### 3.1. Reagents and antibodies

Mouse monoclonal antibodies to PDI (MA3-018 clone RL77, and MA3-019 clone RL90), Pierce BCA Protein Assay Kit, Pierce IP Lysis Buffer, Pierce Control Agarose, Super Signal West Pico Chemiluminescent Substrate, and protease inhibitor cocktail were obtained from Thermo Scientific (Waltham, MA). Mouse monoclonal anti-alphaVbeta3 antibodies clone LM609 (MAB1976), clone 23C6 (CBL544), rabbit polyclonal anti-beta3 antibodies (AB1932, and AB2984), rabbit polyclonal anti-beta1 antibodies (AB1952), mouse monoclonal anti-beta1 antibodies clone P4C10, human plasma fibronectin, vitronectin, fibrinogen and collagen I were purchased from Merck Millipore (Garmstadt, Germany). Goat anti-mouse antibodies, goat anti-rabbit antibodies conjugated with horseradish peroxidase and FAK inhibitor benzene-1,2,4,5-tetrayltetraamine tetrahydrochloride were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse antibodies, goat anti-rabbit antibodies conjugated with horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA). PCMBS (*p*-chloromercuribenzenesulfonic acid) from Toronto Research Chemicals Inc. (Ontario, Canada). Rabbit polyclonal anti-PDI antibodies (ab31811) were purchased from Abcam (Cambridge, UK). Tissue culture reagents, such as fetal bovine serum, PBS buffer and antibiotics were purchased from Invitrogen. DMEM medium was purchased from American Type Culture Collection (Manassas, VA). Avidin-horseradish peroxidase was obtained from Bio-Rad (Hercules, CA). HAT supplement, MPB, avidin-agarose, *N*-ethylmaleimide, quercetin-3-rutinoside, cystamine dihydrochloride, DTNB (5,5'-Dithiobis(2-nitrobenzoic acid), cytochalasin D, RGD peptide, methylcellulose and Src inhibitor 4-(4'-Phenoxyanilino)-6,7-dimethoxyquinazoline, 6,7-Dimethoxy-N-(4-phenoxyphenyl)-4-quinazolinamine were purchased from Sigma-Aldrich (St. Louis, MO). Matrigel was obtained from BD Biosciences (Franklin Lakes, NJ). RGE peptide was from Eurogentec (Liège, Belgium).

### 3.2. EA.hy926 cell culture

The human endothelial cell line EA.hy926, derived by fusion of human umbilical vein endothelial cells with continuous human lung carcinoma cell line A549, was obtained as a gift from C-J Edgell (Pathology Department, University of North Carolina at Chapel Hill). The cells were cultured in high glucose DMEM medium supplemented with 10% fetal bovine serum (v/v), HAT (100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine), and antibiotics. The cell line was cultured at 37 °C in a 90-95% humidified atmosphere of 5% $CO_2$, harvested at confluence with trypsin/EDTA solution and transferred into culture flasks or suspended in appropriate medium before experiments. Cell viability was determined microscopically by Trypan blue exclusion.

### 3.3. Secretion of PDI from endothelial cells

EA.hy 926 cells were plated on ($2\times10^4$ cells/well) 96-well plates coated with poly-L-lysine, collagen type I, fibronectin, fibrinogen or vitronectin (all proteins

at concentration 10 µg/ml), and allowed to attach for 30 minutes. The serum-free medium was collected after 30 minutes. In other experiment endothelial cells were preincubated with mouse monoclonal anti-integrin beta1 (clone P4C10) or mouse monoclonal anti-integrin alphaVbeta3 antibodies (clone LM609) (both at concentration 20 µg/ml) for 10 minutes, and then applied to a plate coated with fibrinogen. In turn, in other experiments the endothelial cells were incubated with RGD or RGE peptides (1 mM) or cytochalasin D (30 µM) or Src or FAK kinase inhibitors for 15 minutes. The serum-free medium was collected after 30 minutes and analyzed by polyacrylamide gel, followed by Western immunoblotting using antibodies specific for PDI (RL77). In some experiments, after collection of the medium, the cells were washed with PBS and lysed with 20 µl/well of Pierce Lysis Buffer. Proteins were separated by SDS-PAGE in 10% gels, transferred to a nitrocellulose and immunodetected by anti-PDI antibodies. The same nitrocellulose was stripped away and blotted with antibodies to beta-actin.

### 3.4. Cell adhesion assay

Adhesion of endothelial cells was performed using 96-well flat-bottom plates coated overnight with poly-L-lysine, fibronectin and fibrinogen (all diluted in PBS to 10 µg/ml). After washing, plates were blocked with 1% BSA in PBS at 37°C in a humidified atmosphere of 5% $CO_2$. Before experiments, the cells were preincubated with a tested reagents, such as bacitracin (100 µM), PCMBS (*p*-chloromercuribenzenesulfonic acid) (100 µM), quercetin-3-rutinoside (100 µM), RGD peptide (1 mM), for 30 minutes at 37°C and 5% $CO_2$. Preincubated and nontreated cells were washed with PBS, harvested and resuspended in serum-free DMEM containing 0.5% BSA. The cells were plated ($2.5 \times 10^4$/well) in wells containing 100 µl serum-free DMEM and allowed to attach for 1.5 hours. After washing away nonadherent cells, the number of adherent cells was determined with the Pierce BCA Protein Assay Kit according to the manufacturer's instructions. The absorbance at 562 nm was measured in a multitask plate reader Victor 2 (PerkinElmer, Waltham, MA).

### 3.5. Wound healing migration assay

The migratory activity of endothelial cells was tested by a "wound healing" assay. The cells were cultured on fibronectin-coated six-well tissue culture plates (Nunc) in a humidified atmosphere containing 5% $CO_2$ at 37 °C. When the monolayer was confluent, a scratch was made using a 200 µl pipette tip. After washing twice with PBS, the cells were incubated with serum-free cell culture medium containing selected factors, such as PCMBS (100 µM), quercetin-3-rutinoside (100 µM), RGD peptide (1 mM), cystamine (100 µM), DTNB (100 µM), or cytochalasin D (30 µM). Images were taken at different time intervals at 10× magnification. After each measurement, the old medium was replaced with a fresh one. The scratch size was measured in four wells per group and analyzed with National Institute of Health (NIH) ImageJ software.

### 3.6. Coimmunoprecipitation and immunoblotting

In some coimmunoprecipitation experiments, before lysis endothelial cells were seeded on tissue culture flasks coated with fibronectin or fibrinogen, and allowed to attach for 1.5. hours in a humidified atmosphere containing 5% $CO_2$ at 37 °C. After washing with PBS, the cells were lysed with Pierce IP Lysis Buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 5% glycerol) supplemented with protease inhibitors cocktail (1 mM PMSF, 0.1 mM EDTA, 1 mM leupeptin, 1 mg/ml aprotinin). The cytoskeletal debris were pelleted for 10 minutes at 14,000×*g* at 4 °C, and the lysates were precleared with 40 µl of Pierce Control Agarose resin for two hours at 4 °C with gentle end-over-end mixing. Subsequently, 500 µg of total precleared cell lysates were incubated overnight with 2 µg of mouse anti-PDI (MA3-018, clone RL77), or rabbit anti-beta3 (AB2984) antibodies at 4 °C with gentle mixing. To collect immune complexes, protein A/G-agarose bead slurry was added, and the incubation was continued for another three hours. The beads were washed five times with lysis buffer, suspended in 2× concentrated Laemmli sample buffer, and boiled for five minutes. Protein detached from beads was separated by SDS-PAGE in 8% gels under reducing and nonreducing conditions, transferred onto nitrocellulose membrane (Bio-Rad), and immunodetected by anti-PDI, or anti-beta3 antibodies, followed by the secondary antibodies conjugated with horseradish peroxidase.

Immunodetection was accomplished by using SuperSignal West Pico Chemiluminescent Substrate and ChemiDoc™ MP Imaging System (Bio-Rad). The protein bands were quantified using ImageJ software.

### 3.7. Coimmunoprecipitation beta1/PDI

The EA.hy926 cells were placed on fibronectin or fibrinogen-coated dishes ($5 \times 10^6$ cells/dish), collected after 1.5 hours and subjected to immunoprecipitation. After washing with PBS, the cells were lysed and 500 µg of total precleared cell lysates were incubated overnight with 2 µg of mouse monoclonal anti-PDI (MA3-018, clone RL77) or rabbit polyclonal anti-beta1 (AB1952) antibodies at 4 °C with gentle mixing. In control experiments, cell lysates were incubated with normal mouse or normal rabbit control IgG, respectively. To collect immune complexes, protein A/G-agarose bead slurry was added, and the incubation was continued for another three hours.

The beads were washed five times with lysis buffer, suspended in 2× concentrated Laemmli sample buffer, and boiled for five minutes. Protein detached from beads was separated by SDS-PAGE in 10% gels under reducing conditions, transferred onto nitrocellulose membrane (Bio-Rad), and immunodetected by anti-PDI, or anti-beta1 antibodies, followed by the secondary antibodies conjugated with horseradish peroxidase. Immunodetection was accomplished by using SuperSignal West Pico Chemiluminescent Substrate and ChemiDoc™ MP Imaging System (Bio-Rad). The protein bands were quantified using ImageJ software.

### 3.8. Confocal microscopy

EA.hy926 endothelial cells were plated at $2\times10^4$ cells per well on Screenstar 96-well microplate (Greiner Bio-One) precoated with poly-L-lysine, fibronectin, or fibrinogen. After incubation for two hours, any non-adherent cells were removed by washing with PBS, and adherent cells were fixed in PHEM buffer (60 mM PIPES, pH 6.9, 25 mM HEPES, 10 mM EGTA, 4 mM MgCl2) containing methanol-free 3% paraformaldehyde for 10 minutes at room temperature. After fixation, the slides were washed with PHEM, and blocked for 60 minutes with 3% BSA in PHEM to avoid nonspecific dye binding. Surface PDI was detected with the affinity-purified rabbit polyclonal anti-PDI antibody (ab31811), and alphaVbeta3 integrin was detected with a mouse monoclonal anti-alphaVbeta3 antibody, clone 23C6 (CBL544) and clone LM609 (MAB1976) for 60 minutes at room temperature in the dark. Antibodies were diluted in PHEM containing 1% BSA to a final concentration of 10 µg/ml.

Subsequently, secondary antibodies (anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 568) were added for one hour at room temperature. After washing with PHEM, cell nuclei were stained using 5 µM Hoechst33342 for 15 minutes. Images were obtained under 6300× magnification using LSM780 microscope equipped with a Plan-Apochromat 63×/1.4. Oil DIC M27 objective, 405 nm laser diode and InTune excitation laser system (Carl Zeiss; Oberkochen, Germany). ZEISS Microscope Software ZEN2012 was used to calculate fluorescence intensities in respective fluorescence channel in pixels within selected region of interest. These values were further used for calculating the coefficient of determination ($r^2$) between the respective channels as a measure of signal co-localization.

### 3.9. Sulfhydryl group labeling

Proteins containing free thiol groups were labeled using the poorly membrane-permeable maleimide reagent MPB (biotinylated 3-*N*-maleimidylpropionyl biocytin). Briefly, MPB (100 µM) was added to endothelial cells washed in serum-free medium, and incubated for 30 minutes at room temperature. The labeling reaction was quenched by addition of GSH (200 µM) and iodoacetamide (400 µM), with incubation for 10 minutes. The specificity of MPB labeling for free thiol groups was shown by pretreating the endothelial cells with the sulfhydryl reagent *N*-ethylmaleimide (1 mM). MPB-labeled proteins were precipitated with avidin-agarose subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. Biotinylated proteins were detected with avidin-horseradish peroxidase using chemiluminescence. In some studies, proteins labeled with MPB were immunoprecypitated with anti-PDI (MA3-018, clone RL77) or anti-beta3 antibodies (AB2984) at a concentration of 10 µg/ml, coupled with protein A/G-agarose beads, with overnight incubation at 4 °C with gentle mixing. Samples were analyzed by SDS-PAGE and immunoblotting as describe above.

### 3.10. *In vitro* angiogenesis assay - endothelial cell spheroids

Endothelial cell spheroids containing about 750 cells were generated overnight by incubating cells in 0.25% methylcellulose in culture medium supplemented with 20% FBS in 96-well nonadherent U-bottomed plates (Costar).

Subsequently, the spheroids were embedded into collagen gels. A collagen stock was prepared before use by mixing acidic type I collagen from rat tails (equilibrated to 3 mg/ml) with 10× concentrated DMEM medium and 0.1. N NaOH to adjust the pH to 7.4. This solution (500 µl) was mixed with 500 µl room temperature DMEM basal medium containing 40% FBS, 1.2% methylcellulose, and about 50 spheroids. All steps were performed in a cold ambiance to avoid premature collagen gel polymerization. The spheroid-containing gel was rapidly transferred into 24-well plates and allowed to polymerize for 30 minutes at 37 °C. Afterwards, 200 µl DMEM basal medium containing TNF-alpha (in a final concentration of 100 ng/ml), or TNF-alpha plus tested reagents (PCMBS, QR (quercetin-3-rutinoside), cystamine, DTNB,) was pipetted on top of the gel. The gels were incubated in a humidified atmosphere containing 5% $CO_2$ at 37 °C for three days. Spheroid formation was observed at 40× magnification using inverted microscope Olympus CKX41 and images were captured with an attached Olympus C3040 camera.

### 3.11. Statistical analysis

The statistical significance of the differences between the experimental conditions was determined by Student's *t* test for unpaired groups. *p* values less than 0.05 were considered significant. Densitomerty of Western Blots was performed using National Institute

of Health (NIH) ImageJ software. Data were analyzed using GraphPad Prism software.

## 4. RESULTS

### 4.1. Secretion of PDI from endothelial cells

The role of integrins in secretion of PDI from the endothelial cells was examined. We assumed that upon activation of endothelial cells, PDI is released from cells and associates with integrin receptors on the cells surface, where it maintains the receptor in its active state. Although our previous observations indicated that PDI is constitutively released from endothelial cells, the aim of the next stage was to confirm whether the adhesion of endothelial cells to extracellular matrix proteins is associated with an increase of PDI secretion. For this purpose, the EA.hy926 cells were plated on 96-well plates coated with poly-L-lysine, collagen, fibronectin, fibrinogen or vitronectin and allowed to attach for 30 minutes. The serum-free medium was collected at specific time points and PDI was detected by immunoblotting with antibodies for PDI. During adhesion to fibronectin, fibrinogen or vitronectin of up to 30 minutes, a significant increase in PDI release from endothelial cells was observed compare to the cells adhering to poly-L-lysine or collagen (Figure 1).

Participation of the integrins in signal transduction leading to secretion of PDI from endothelial cells was confirmed by incubation of the cells with RGD peptide and as control with RGE peptide. The cells were preincubated with peptide RGD or RGE for 10 minutes, and then applied to a plate coated with fibronectin or fibrinogen. After 5, 15 and 30 minutes, the collected serum free medium was analyzed by polyacrylamide gel, followed by Western immunoblotting using antibodies specific for PDI (RL77). RGD peptide was found to have a strong inhibitory effect on the secretion of PDI (Figure 2A).

Therefore the signaling pathways were examined to determine the role of integrin in the process of PDI secretion from endothelial cells. The endothelial cells were preincubated with Src and FAK kinase inhibitors and allowed to adhere to fibronectin or fibrinogen. The media were collected at specific time points and PDI concentration detected by immunoblotting.

Figure 2B shows that the inhibition of Src and FAK kinases during adhesion to fibronectin or fibrinogen resulted in a decreased amount of PDI released from endothelial cells.

Previous studies showed that PDI is a novel regulator of actin cytoskeletal organization through thiol-disulfide rearrangement in the beta-actin molecule in response to integrin activation (11). The adhesion of endothelial cells to extracellular matrix proteins leads to the activation of integrins accompanied by the reorganization of actin cytoskeleton. Therefore, in the next stage of the study we examined whether the reorganization of the actin cytoskeleton influences the secretion of PDI from endothelial cells. The cells were pre-incubated with the actin-disturbing reagent cytochalasin D for 15 minutes then the cells were allowed to attach to fibronectin. Subsequently, the medium was collected at appropriate intervals up to 60 min and analyzed by immunoblotting with anti-PDI antibody. Destruction of the actin cytoskeleton during adhesion of endothelial cells to fibronectin in the presence of cytochalasin D resulted in reduced PDI secretion (Figure 2C).

To compare the effect of beta1 integrin with beta3 integrin on secretion of PDI, the endothelial cells were preincubated with anti-integrin beta 1 (clone P4C10) or anti-integrin alphaVbeta3 antibodies (clone LM609) for 10 minutes, and then applied to a plate coated with fibrinogen. The serum-free medium was collected after 30 minutes and analyzed by polyacrylamide gel, followed by Western immunoblotting using antibodies specific for PDI (RL77). Proteins were separated by SDS-PAGE in 10% gels, transferred to a nitrocellulose and immunodetected by anti-PDI antibodies. The same nitrocellulose was stripped away and blotted with antibodies to beta-actin (Figure 2D). Inhibition of activation of alphaVbeta3 integrin by monoclonal antibody, clone LM609, cause decrease secretion of PDI from endothelial cells but antibodies anti beta1 have less effect on inhibiting secretion of PDI. These results indicate to a dominant role of alphaVbeta3 integrins in activation of signaling pathways leading to release of PDI during endothelial cells activation. Despite that these two integrins make a complex with PDI, we are observed enhanced effect of adhesion endothelial cells to fibrinogen or fibronectin on creation of complexes between PDI and alphaVbeta3 integrin compare to beta1 integrin (data not shown).

### 4.2. Association of PDI with αvβ3 integrin on endothelial cells during adhesion

A previous studies have determined that PDI is directly associated with alphaVbeta3 integrin on endothelial cells (4). In the next stage we attempted to determine whether adhesion increases the specific interaction between PDI and alphaVbeta3. For this purpose we performed coimmunoprecipitation experiments. The EA.hy926 cells were placed on fibronectin or fibrinogen-coated dishes, collected after 1.5 hours and subjected to immunoprecipitation. To identify the direct interaction between PDI and alphaVbeta3 integrin, the cell extracts were incubated with antibodies to alphaVbeta3 or antibodies to PDI, respectively.

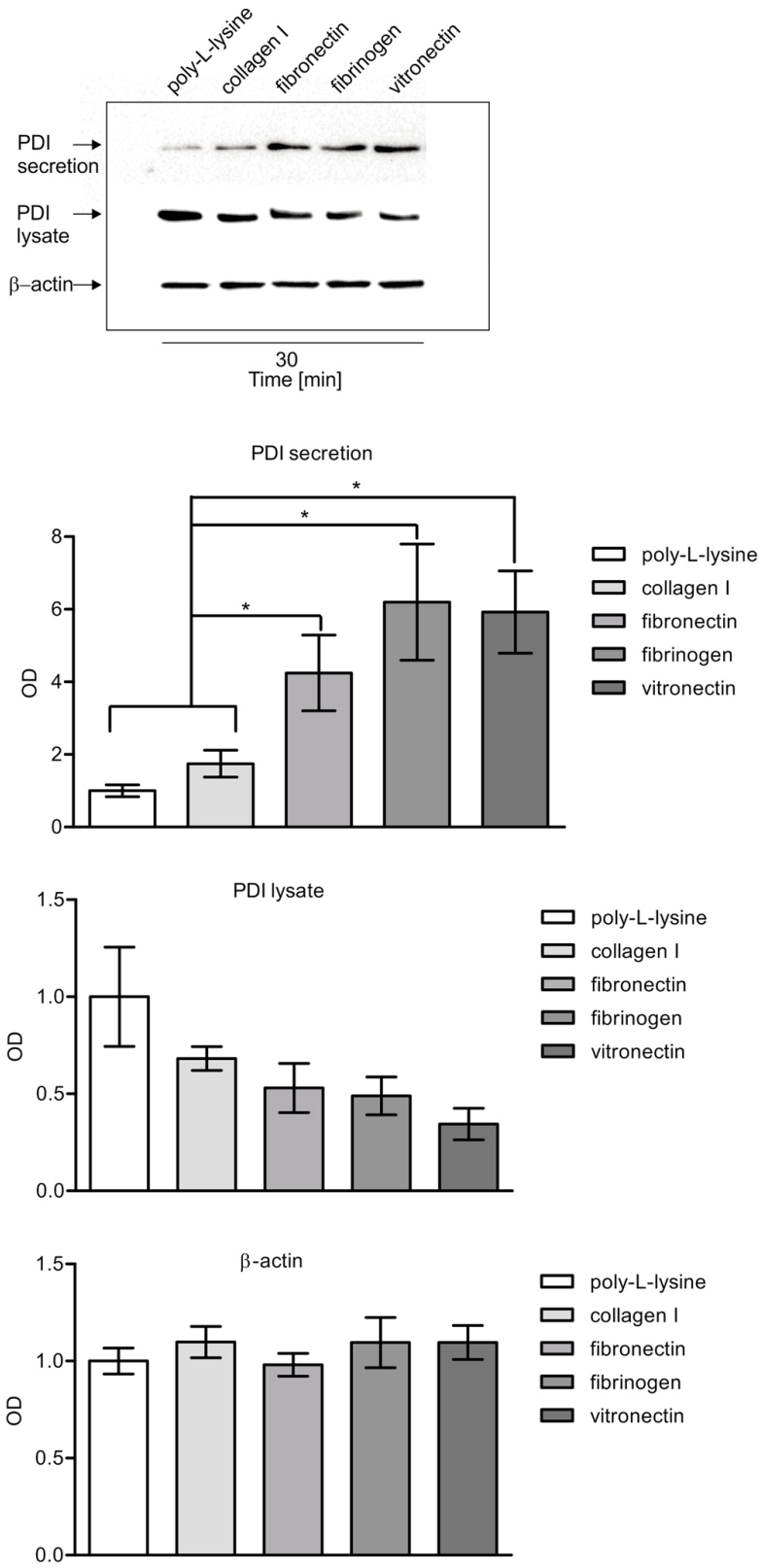


**Figure 1.** Secretion of PDI from EA.hy926 cells during adhesion**.** Secretion of PDI from EA.hy926 endothelial cells seeded on 96-well plates precoated with poly-L-lysine, collagen I, fibronectin, fibrinogen and vitronectin. After 1.5 h attachment cells were washed with PBS and 30 µl serum-free medium was added to each well. The medium was collected and analyzed by 10% SDS-PAGE followed by immunoblotting with anti-PDI antibody (MA3-018, clone RL77). Endothelial cells which adhere to fibronectin or fibrinogen release more PDI compare to collagen I and control (poly-L-lysine). Differences in secretion of PDI were estimated using densitometry analysis using National Institutes of Health (NIH) ImageJ.

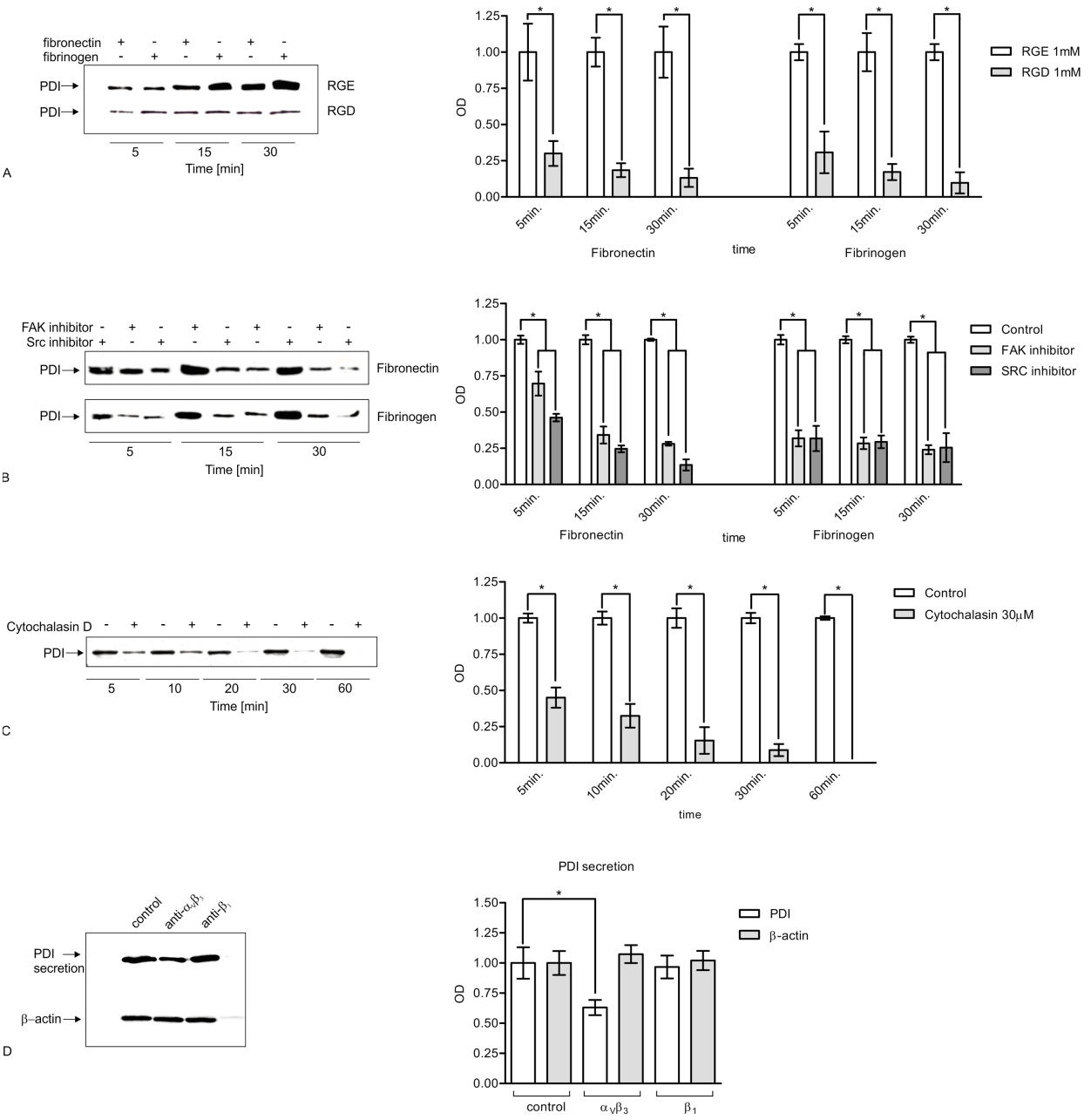


**Figure 2.** Activation of integrin mediates PDI secretion from EA.hy926 cells**.** A. Secretion of PDI from EA.hy926 endothelial cells seeded on 96-well plates precoated with fibronectin or fibrinogen in the presence of RGD or RGE peptides. B. Secretion of PDI from EA.hy926 endothelial cells seeded on 96-well plates precoated with fibronectin or fibrinogen in the presence of Src and FAK kinases inhibitors. C. EA.hy926 endothelial cells seeded on 96-well plates precoated with fibronectin in the presence of cytochalasin D. As shown in panels A, B and C secretion of PDI from EA.hy926 endothelial cells was inhibited by RGD peptide, Src and FAK kinase inhibitors and cytochalasin D respectively. The immunoblot shown in each panel is representative of three separate experiments. To compare the effect of integrin beta1 with integrin beta3 on secretion of PDI, the EA.hy926 cells were preincubated with mouse monoclonal anti-integrin beta1 or mouse monoclonal anti-integrin alphaVbeta3 antibodies and then applied to a plate coated with fibrinogen. The serum-free medium was collected analyzed by polyacrylamide gel, followed by Western immunoblotting using antibodies specific for PDI. The same nitrocellulose was stripped away and blotted with antibodies to beta-actin. D. Antibody anti beta3 is more effective and inhibits release of PDI compare to beta1 antibody from endothelial. The immunoblot shown in each panel is representative of three separate experiments.

Specifically-bound proteins were solubilized in Laemmli sample buffer and separated by SDS-PAGE under nonreducing and reducing conditions using 10% running gel. Both proteins were identified by Western immunoblotting of the immunoprecipitates using specific antibodies: the presence of PDI in complex with alphaVbeta3 was evidenced by blotting with antibodies to PDI when the immunoprecipitate was pulled down with antibodies to alphaVbeta3, and blotting with antibodies to alphaVbeta3 when the

immunoprecipitate was pulled down with antibodies to PDI. In control experiments, cell lysates were incubated with normal mouse or normal rabbit control IgG, respectively.

No PDI or alphaVbeta3 was found in immunoprecipitates pulled down with control antibodies after blotting with antibodies to PDI or alphaVbeta3. PDI was found to be present in a high molecular complex with the alphaVbeta3 integrin. A single band with a molecular mass of 140 kDa was observed under non-reducing conditions. After reduction, two components were found corresponding to the PDI (50 kDa) and beta3 subunit integrin (90 kDa). The control cells placed on poly-L-lysine-coated dishes contained a slight amount of PDI- alphaVbeta3 integrin complexes (Figure 3A, B).

Our experiments showed that the number of PDI- alphaVbeta3 complexes significantly increased after adhesion of endothelial cells to fibronectin or fibrinogen, compared to adhesion to poly-L-lysine. PDI-alphaVbeta3 complexes observed in endothelial cells adhering to poly-L-lysine might indicate the presence of a transient form of the alphaVbeta3 integrin in the integrin population on the endothelial cell surface. These forms of alphaVbeta3 integrin were associated with PDI without activation. Previous studies have shown that the population of alphaIIbbeta3 integrin isolated from platelets was heterogeneous: 10% of the alphaIIbbeta3 molecules were active and displayed the ability to bind the RGD peptide, 15% were in complexes with actin, and the rest of the alphaIIbbeta3 molecules were in an inactive conformation with low ligand-binding affinity (14). Our results suggest that the alphaVbeta3 integrin population in endothelial cells may have similar heterogeneity.

Subsequently, spatial association between PDI and integrin alphaVbeta3 molecules on the cell surface was confirmed by verifying their co-localization under a confocal microscope. Figure 4A shows that on poly-L-lysine, while both types of molecules are expressed on cell surface, the degree of their co-localization is very small. However, even a short (2h) period of contact with fibronectin or fibrinogen led to a significant increase of co-localization between PDI and total integrin alphaVbeta3.

This effect was visible qualitatively in microscopic images, but could also be reliably quantified by calculating correlation between staining in both channels (Figure 4B). The increase in co-localization was even more prominent when an antibody detecting exclusively the active form of integrin alphaVbeta3 (LM609) was used (Figure 4C, D). Apart from a significantly higher correlation, particularly strong co-localization of these two proteins can clearly be seen in lamellipodial protrusions, suggesting that this is where PDI participates in maintaining integrin in its active form during adhesion (Figure 4C).

To evaluate the role of free thiol groups in interaction of PDI with alphaVbeta3 integrin during adhesion, EA.hy926 cells were treated with membrane-impermeable biotinylated 3-*N*-maleimidylpropionyl biocytin (MPB), then lysed and examined using SDS-PAGE and immunoprecipitation. The cells were labeled with 100 μM MPB in serum-free DMEM for 30 minutes at room temperature, before being stopped by addition of glutathione (GSH) and iodoacetamide followed by incubation for 10 min. After being washed with serum-free DMEM, the cells were lysed and MPB-containing proteins were isolated with avidin-agarose, washed and solubilized in Laemmli sample buffer. After separation by 10% SDS-PAGE and transfer to nitrocellulose, biotinylated proteins were detected with avidin conjugated with horseradish peroxidase. The number of proteins contains free thiol groups significantly increased in endothelial cells which adhere to fibronectin or fibrinogen (Figure 5A).

In the second part of the experiment, MPB-containing proteins were immunoprecipitated with anti-PDI or anti- alphaVbeta3 antibodies coupled with protein A/G-agarose beads, followed by blotting with avidin-HRP. The presence of free thiol groups was significant in both analyzed proteins in EA.hy 926 cells adhered to fibronectin or fibrinogen (Figure 5B). In EA.hy 926 cells seeded to poly-L-lysine, PDI or alphaVbeta3 integrin did not contain free thiol groups. To confirm the specificity of MPB labeling, the cells were preincubated with the sulfhydryl reagent N-ethylmaleimide before reaction with MPB in parallel experiments. Proteins in such samples were not stained with MPB, showing that the appearance of free thiol groups was dependent upon adhesion and their detection was specific (data not shown). These experiments support the role of free thiol groups in interaction between PDI and alphaVbeta3 integrin in adhering endothelial cells. After integrin activation, PDI is released from endothelial cells and then associates with integrin by disulfide bonds.

### 4.3. Influence of PDI inhibitors and thiol group blockers on the functions of endothelial cells

A series of experiments involving cell adhesion, migration and spheroid formation was performed to examine the influence of PDI inhibitors (quercetin-3-rutinosite, bacitracin), thiol group blockers (PCMBS, cystamine, DTNB) on the functions of endothelial cells (EA.hy926).

Previous studies showed that surface sulfhydryls and extracellular PDI are important for integrin-mediated platelet adhesion (12,13) and activation of alphaVbeta3 integrin in endothelial cells

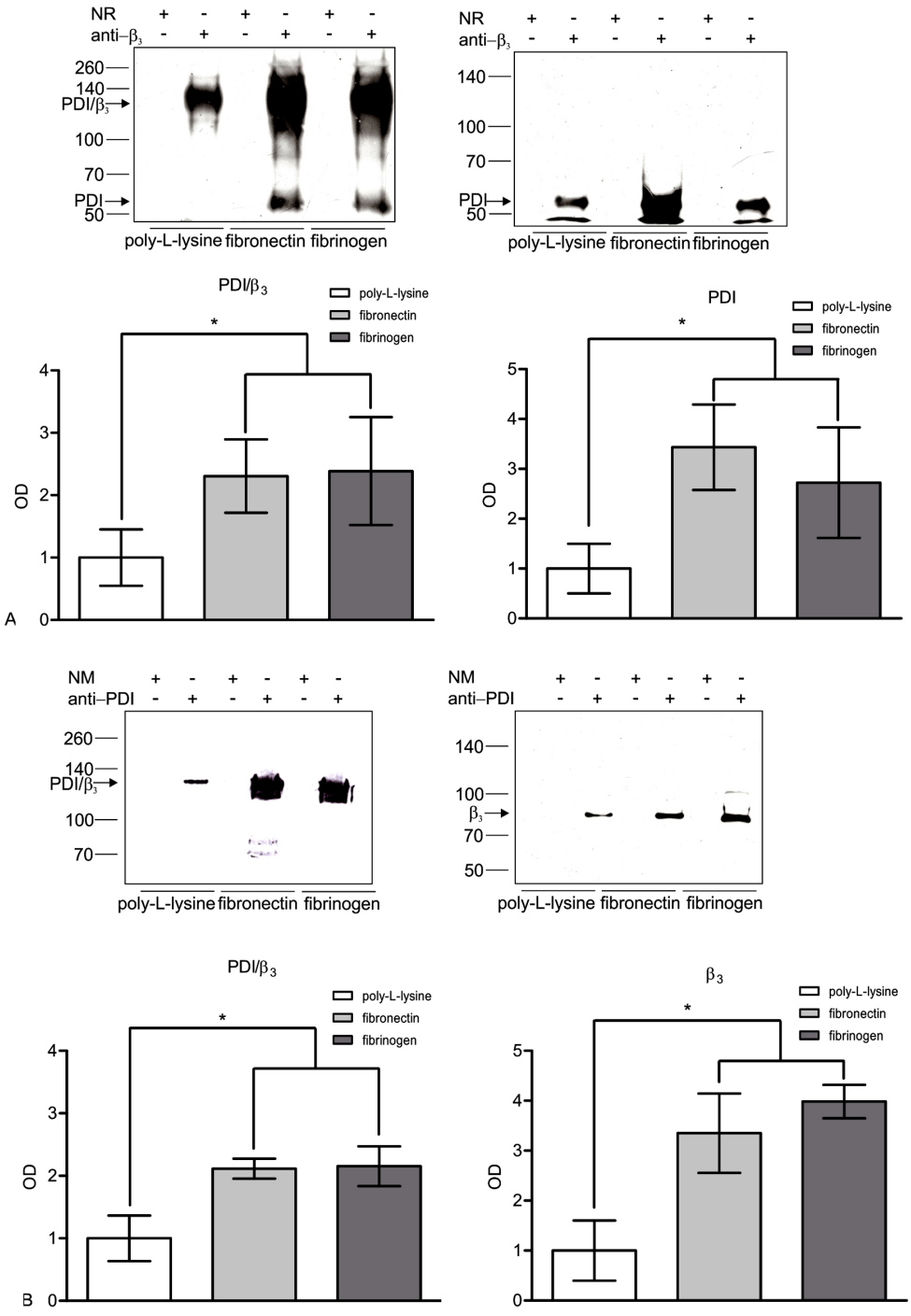


**Figure 3.** Increase association of PDI with alphaVbeta3 integrin in EA.hy926 endothelial cells adhering to extracellular matrix. EA.hy926 endothelial cells attached to poly-L-lysine, fibronectin or fibrinogen were lysed and processed for immunoprecipitation of PDI or alphaVbeta3 integrin by agarose resin and specific anti-alphaVbeta3 or anti-PDI antibodies followed by electrophoresis under non reducing (left panels A and B) and reducing conditions (right panels A and B) and immunoblotting using anti-PDI (panel A), or anti-beta3 (panel B) antibodies. Panel A left: Immunoblotting of PDI with alphaVbeta3 integrin in a high molecular weight complex approximately 140kDa where the immuneprecipitates were probed using anti-alphaVbeta3 antibody. Increase of PDI and alphaVbeta3 integrin complex is shown in lanes when EA.hy926 endothelial cells were grown on fibronectin or fibrinogen. Panel A right: Immunoblotting of PDI as detailed above in left panel A except that were performed under reducing conditions. Lower bands of approximately 50 kDa indicate PDI increase when EA.hy926 endothelial cells were grown on fibronectin or fibrinogen. Panel B left: Immunoblotting of alphaVbeta3 integrin with PDI in a high molecular weight complex approximately 140kDa. Increase of PDI and alphaVbeta3 integrin complex is observed when the immunoprecipitates were probed using anti-PDI antibody. Increase of PDI and alphaVbeta3 integrin complex is shown in lanes when EA.hy926 endothelial cells were grown on fibronectin or fibrinogen. Panel B, right: Immunoblotting of alphaVbeta3 integrin as detailed above in panel B left, except that were performed under reducing conditions. Increased alphaVbeta3 integrin bands of approximately 90 kDa are observed when EA.hy926 endothelial cells were adhered into fibronectin or fibrinogen. In control samples, immunoprecipitates were probed using normal mouse (NM) or normal rabbit (NR) antibodies (panels A and B, lanes 1, 3 and 5). The results shown in panels A and B are representative of three experiments.

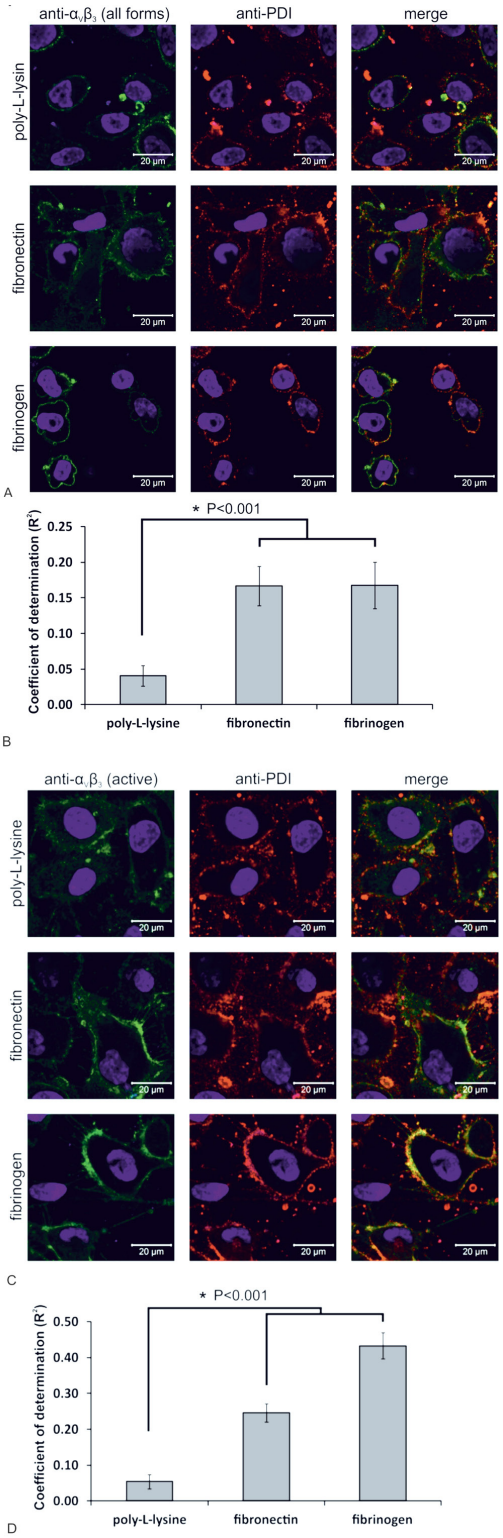


**Figure 4.** Increased co-localization of PDI with alphaVbeta3 integrin in EA.hy926 endothelial cells adhering to extracellular matrix. EA.hy926 cells were seeded on poly-L-lysine, fibronectin or fibrinogen pre-coated surface and were left to adhere for 2h. Subsequently, cells were fixed but not permeabilized and epitopes of PDI and integrin alphaVbeta3 accessible from the outside of the cell were immunostained with primary antibodies against integrin alphaVbeta3 (clone 23C6, detecting all forms of integrin alphaVbeta3 – panels A, B - and clone LM609, detecting only the active form of integrin alphaVbeta3 – panels C, D) and PDI (clone ab31811) as well as respective secondary antibodies conjugated to Alexa Fluor 488 (integrin alphaVbeta3) or Alexa Fluor 568 (PDI). Cell nuclei were counterstained with Hoechst 33342 dye (blue color). (A, C) Representative confocal images are presented to show fluorescence distribution in single fluorescence channel of stained integrin alphaVbeta3 (green color) and PDI (red color) or in both channels (merged image – yellow color indicates co-localization). Scale bar: 20 µm. (B, D) Coefficient of determination ($R^2$) between green and red fluorescence in pixels was calculated for several single-cell regions of interest and is presented as mean ± S.D. (n = 6). Asterisk (*) indicates significant differences at $p$ <0.05.

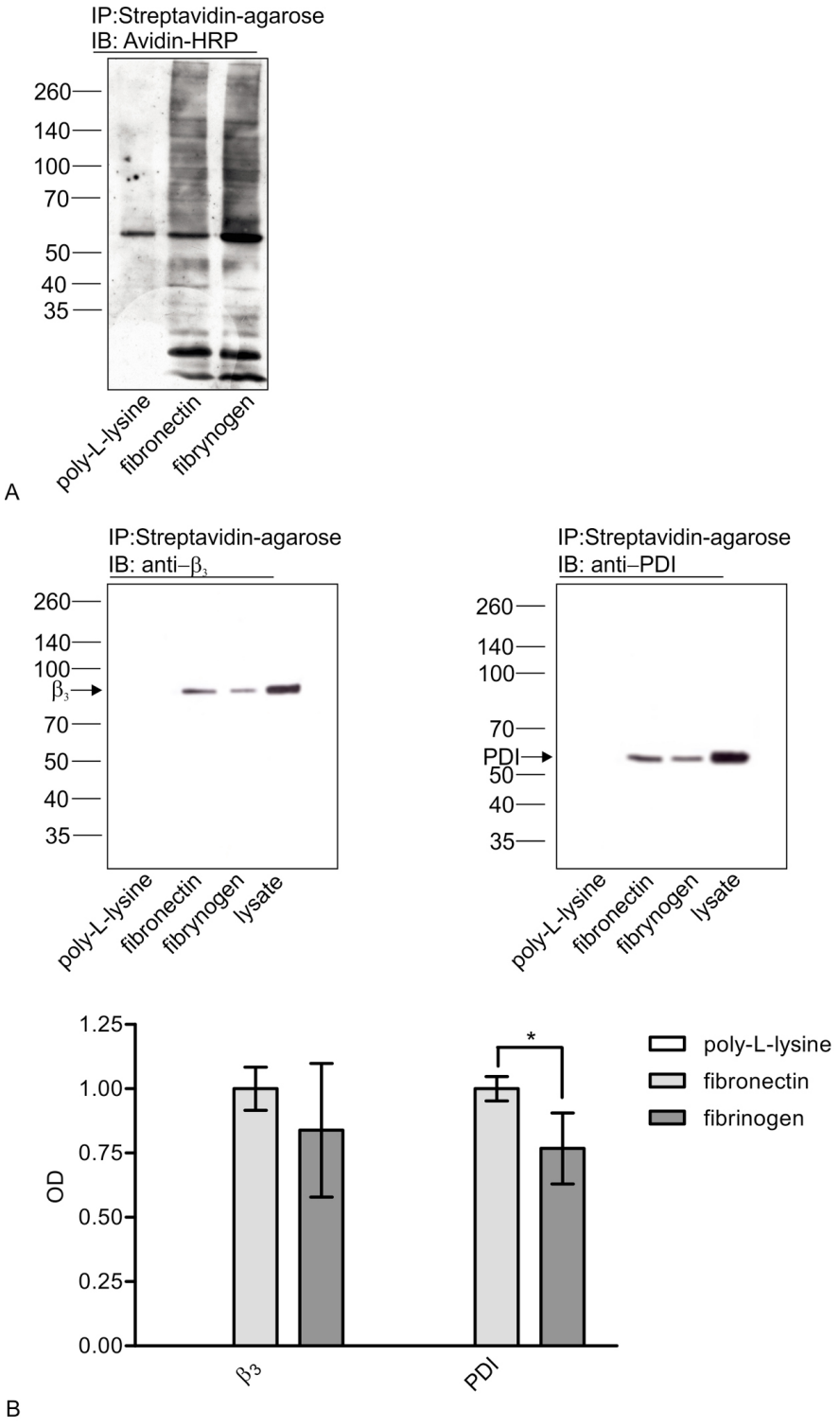


**Figure 5.** Generation of free thiol groups in PDI and alphaVbeta3 molecules during adhesion of EA.hy926 cells. (A) EA.hy926 cells attached to poly-L-lysine, fibronectin or fibrinogen were labeled with MPB and extracted with lysis buffer. MPB-labeled proteins were precipitated with avidin-sepharose, washed and solubilized in Laemmli sample buffer. After separation by SDS-PAGE and transfer to nitrocellulose, biotinylated proteins were visualized by avidin conjugated with horseradish peroxidase. The number of proteins contains free thiol groups significantly increased in endothelial cells which were adhered to fibronectin (lane 2) and fibrinogen (lane 3). (B) The proteins labeled with MPB were immunoprecypitated (IP) with antibodies to alphaVbeta3 (left panel) and to PDI (right panel), coupled with protein A/G-agarose beads. Samples were analyzed by SDS-PAGE and immunoblotting (IB) as describe above. In left panel the arrow points to a alphaVbeta3 integrin of approximately 90 kDa and in the right panel the arrow points PDI band of approximately 50 kDa. As shown in both panels the presence of free thiol groups are significant in both analyzed proteins in EA.hy 926 cells which were adhered to fibronectin (lanes 2) or fibrinogen (lanes 3), no bands are observed when EA.hy 926 cells were adhered to the poly-L-lysine (lanes 1). The immunoblot shown in each panel is representative of three separate experiments.

(4). A series of functional experiments was performed to verify the role of disulfide bond exchange in endothelial cell function.

In adhesion assay EA.hy926 cells were pre-incubated with PDI inhibitors such us bacitracin, quercetin-3-rutinosite or a thiol group blocker - PCMBS or RGD peptide. The endothelial cells pretreated with tested reagents had a clearly lower level of adhesion than untreated cells (Figure 6A). Incubation of endothelial cells with membrane-impermeant sulfhydryl group reagents PCMBS resulted in significant inhibition of adhesion to fibronectin or fibrinogen, indicating that ecto-sulfhydryl blocker neutralizes the activity of the integrins. Similarly, PDI inhibitors such as quercetin-3-rutinoside and bacitracin effectively diminish endothelial cell adhesion to extracellular matrix proteins. This confirms earlier observations that the reorganization of disulfide bonds in integrins is essential in the process of adhesion (12).

To examine the effect of thiol group blockers and PDI inhibitors on the migratory ability of endothelial cells, a “wound healing” assay was performed. Cells were incubated with PDI inhibitor (quercetin-3-rutinoside), thiol group blockers (PCMBS, cystamine, DTNB), RGD peptide or cytochalasin D. The tested reagents were found to decrease migratory activity of endothelial cells (Figure 6B).

To evaluate the functional importance of PDI and thiol-disulfide balance on angiogenic properties of endothelial cells, spheroid-sprouting assay (Figure 6C) were applied. In the spheroid-sprouting assay, endothelial cells were generated in culture medium, and subsequently embedded into collagen gels. The addition of 100 ng/ml TNF-alpha stimulated capillary sprouting and increased both the number and the lengths of capillary sprouts. Without cytokine stimulation, only a few short capillary sprouts grew out from cells, whereas addition of PDI inhibitors, thiol group blockers, RGD peptide and cytochalasin D completely blocked the formation of cytokine-dependent capillary sprouts (Figure 6C). Our findings demonstrate that PDI has an important role in endothelial cell adhesion, migration and tubular formation which supports earlier observations on the role of PDI in endothelial cell function. The observations indicate to the role of secreted PDI on functions of endothelial cells.

## 5. DISCUSSION

Our findings indicate that activation of alphaVbeta3 integrin by adhesion results in the secretion of PDI from endothelial cells. The application of RGD peptide, Src and FAK kinase inhibitors and anti alphaVbeta3 antibodies confirmed that integrin activation was involved in the secretion of PDI from endothelial cells. In addition, treatment of the cells with an actin cytoskeleton-disrupting agent, cytochalasin D, confirmed that cytoskeleton reorganization plays a role in the process of PDI secretion.

Co-localization of PDI with integrin alphaVbeta3 in confocal images is a strong indication of complex formation between these molecules, particularly since we were able to observe a significant increase in the degree of this co-localization in cells adhering to extracellular matrix proteins which interact with integrin alphaVbeta3 (fibronectin, fibrinogen). Moreover, when applying a more specific antibody that binds exclusively to the active conformation of alphaVbeta3, we saw even higher values of coefficient of determination (indicating higher correlation and hence more specific co-localization), confirming the correlation of this complex formation with increased integrin functionality in adhesion. Furthermore, the cellular localization of this co-localized signal to lamellipodia-like structures added more weight to our conclusion that the studied protein-protein interaction has a specific functional role in adhesion-related cell surface phenomena. A higher number of complexes between PDI and alphaVbeta3 integrin was found in adhering endothelial cells, as indicated by immunoprecipitation. In addition, the adhesion of endothelial cells to extracellular ligands was found to induce the formation of free thiol groups within the PDI and alphaVbeta3 molecules. The thiol-disulfide balance influences the adhesive potential of endothelial cells. Thiol group blockers reduce adhesion, migration and spheroids structure formation. Our results demonstrate that endothelial cell activation increases the release of PDI to extracellular space. Number of endothelial cell agonists such as thrombin, histamine and PMA have been shown to stimulate the release of PDI from endothelial cells (3,4). Our data represent the first confirmation that integrin activation during adhesion is a cause of secretion of PDI from the endothelium.

Earlier reports indicate that PDI is secreted by endothelial cells and is deposited on the cell surface (5,16). The release of PDI from active endothelial cells may suggest the involvement of secretory granules. Although PDI is an abundant protein of the endoplasmic reticulum, it is also located in granular compartments outside the ER in endothelial cells. PDI colocalizes with GRO-alpha in small secretory granules but does not localize in Weibel–Palade bodies (3,17).

PDI is also involved in thrombus formation (3,4). The translocation of PDI towards the plasma membrane and its subsequent release from endothelial cells is necessary for thrombus formation. After laser-induced vascular injury, PDI was rapidly released from endothelial cells before secretion from the bound platelets, and then accumulated at the site of injury (3,18). Kim *et al.* (19) report that after laser-induced arteriolar injury, platelet PDI is important for platelet

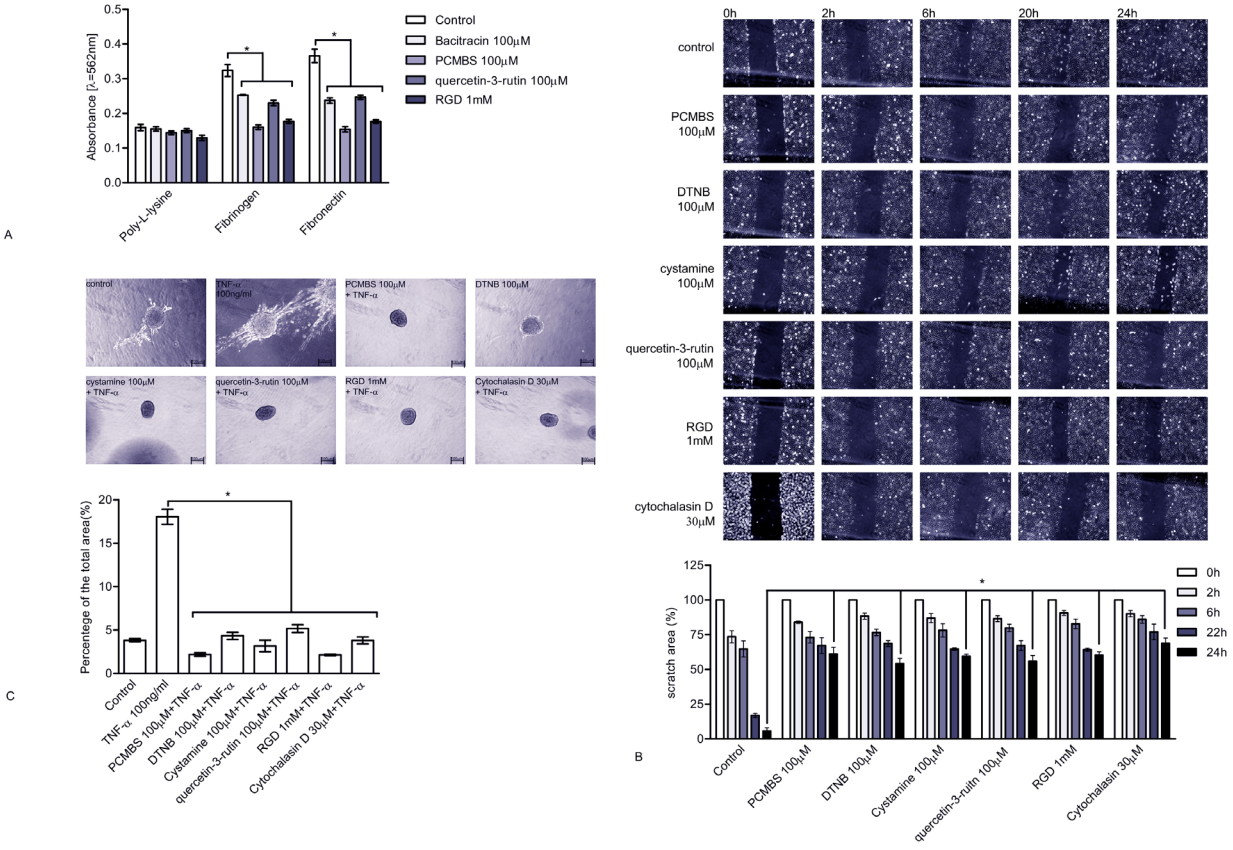


**Figure 6.** Thiol group blockers and PDI inhibitors decrease adhesion and migration of EA.hy926 cells. A. Adhesion of EA.hy926 endothelial cells to immobilized poly-L-lysine, fibrinogen or fibronectin in the presence of PDI inhibitors (bacitracin and quercetin-3-rutin), thiol group blockers pCMBS and RGD peptide. Poly-L-lysine (control), fibrinogen or fibronectin were immobilized onto 96 well plates. Preincubated with bacitracin (100 μM), quercetin-3-rutin (100 μM), PCMBS (100 μM) and RGD peptide (1mM) or nontreated EA.hy926 endothelial cells were applied onto the poly-L-lysine, fibrinogen or fibronectin matrix and incubated for 90 min at 37 °C. Following extensive washings of each well with PBS, total amount of adherent EA.hy926 endothelial cells was determined by the BCA method as detailed in the Material and Methods section. Levels of adherent EA.hy926 endothelial cells to the fibrinogen or fibronectin were higher compared to the poly-L-lysine (control). In the poly-L-lysine coated plate no inhibition of adhesion of EA.hy926 endothelial cells in the presence of bacitracin, quercetin-3-rutin, PCMBS and RGD peptide was observed. When fibrinogen or fibronectin were used as a matrix inhibition of adhesion of EA.hy926 endothelial cells preincubated with bacitracin, quercetin-3-rutin, PCMBS and RGD peptide was found to be significantly different from the nontreated EA.hy926 (control). Numbers represent the mean +SEM of three separate experiments. The asterisk (*) indicates significant differences at the $p$ <0.001. B. Wound healing assay: Inhibition of the migration of EA.hy926 endothelial cells on fibronectin-coated plates by thiol group blockers (PCMBS, DTNB, cystamine), quercetin-3-rutin PDI specific inhibitor, RGD peptide and cytochalasin D. The cells were cultured on fibronectin-coated 6-well plates, and after reaching confluency, a scratch was made. Movement of cells into wound area was shown at 0, 2, 6, 12 and 24 h post scratch. Images were taken at different time intervals using inverted microscope Olympus CKX41 combined with Olympus C3040 camera. The area of the scratch size was measured using National Institutes of Health (NIH) ImageJ program with MiToBo plugin. In control (top panel) migrated EA.hy926 endothelial cells covered whole wounded area after 24h. Inhibition of the migration of EA.hy926 endothelial cells by thiol group blockers (PCMBS, DTNB, cystamine), quercetin-3-rutin PDI specific inhibitor, RGD peptide and cytochalasin D was observed in 2, 6, 12 and 24 h after wounding (lower panels). Quantitation of the scratch size of migrating EA.hy926 cells (right panel). Cell migration was assessed by recover of the scratch. The area of the wound was measured at the four time points in seven groups. The results were expressed as % reduction of initial scratch area in each group. Values are the means ±SEM for 4 separate wells in which wounding was performed at each time point. Significance at *$p$ < 0.001, compared seven different groups at 24h. C. Thiol groups blocker and PDI inhibitor decrease spheroid structure formation. Endothelial cell spheroids formation. Endothelial cell spheroids formation in collagen gel was visualized by Olympus CKX41 microscope with Olympus C3040 camera. Cell spheroids were generated overnight in 0.25% methylcellulose in culture medium supplemented with 20% FBS in 96-well non adherent U-bottomed plates embedded into collagen gels. Subsequently, tested reagents were pipetted on top of gels. Capillary sprouting originating from the spheroids was quantified (lower panel). Spheroidal structures area was measured as a percentage of the total area using ImageJ program. Addition of 100 ng/ml TNF-alpha stimulates capillary sprouting, increasing both the number and the length of capillary sprouts compared to the control (untreated). Without cytokine stimulation, only a few short capillary sprouts grew out from cells, whereas addition of PDI inhibitors, thiol group blockers, RGD peptide and cytochalasin D completely blocked cytokine-dependent capillary sprouts forming. Numbers represent the mean +SEM of three separate experiments. *, $p$ <0.001 compared to corresponding control. Representative images of three independent experiments are shown. Bars, 100 μm.

accumulation but not for initial adhesion and fibrin generation. Although both platelets and endothelial cells are important sources of PDI during thrombus formation, the endothelium is the initial source of PDI. Released PDI is captured by endothelial alphaVbeta3 and platelet alphaIIbbeta3 (5,20). The protein disulfide isomerase mediates in the transition of integrin to a ligand-competent state, and this can act as an alternative (non-canonical) mechanism in integrin activation. *In vivo* studies have confirmed that

while PDI plays an essential role in platelet thrombus formation and fibrin generation (3,4,17,18,21), lack of alphaVbeta3 in the endothelium fails to generate a thrombus (20).

Recent observations support the critical role of alphaVbeta3 in the initiation of pathway to fibrin formation. Cho *et al.* observed a significant decrease of PDI accumulation in the arteriolar wall after injury induced by laser in beta3 null mice (20). This can lead to the conclusion that beta3 integrin activation is dispensable in the secretion and accumulation of PDI and consequentially, in thrombus formation. Similarly, our own results indicate that activation of integrin during adhesion cells to extracellular matrix proteins triggers signals, thus inducing the secretion of PDI from endothelial cells. Our data have shown that endothelial cells which adhere to fibrinogen or fibronectin release more PDI than endothelial cells which adhere to collagen and poly-L-lysine. PDI secretion was inhibited by RGD peptide, cytochalasin D, Src and FAK kinase inhibitors and anti alphaVbeta3 antibody, which indicates that beta3 integrin may participate in this process. The role of PDI in integrin activation was confirmed by functional assays, where free thiol group blockers and PDI inhibitor have reduced adhesion, migration and spheroids formation. A previous study found that knockdown of PDI in HUVEC reduces migration, adhesion and capillary tube formation (22).

The activation of integrin leads to conformational changes resulting in the creation of a spatial form capable of binding a ligand. The integrin is closely associated with the actin cytoskeleton. Extracellular signals are carried into the cells by the integrin, where it triggers a cascade of factors involved in protein phosphorylation, and cytoskeletal reorganization (23). It has been shown that PDI is a novel regulator of actin cytoskeletal organization through reorganization of the disulfide bonds in the beta actin molecule in response to integrin activation. During cell attachment, the signal is transduced into the cell, which finally leads to the translocation of PDI from the subcellular compartments to the inner layer of the plasma membrane, and then to extracellular space. This may be one of the mechanisms leading to the movement of PDI into the extracellular membrane (12).

The role of actin cytoskeleton disorganization in the expression of PDI on the surface was demonstrated in endothelial cells during DENV infection (25). The occurrence of PDI on the endothelial cell surface is mediated through the KDEL receptor and activation of Src family kinase (23). Earlier reports note that the PDI present on the surface of exocrine pancreatic cells, hepatocytes and platelets contains the KDEL retention sequence (25,26,27).

Our results indicate that the PDI released from endothelial cells binds dominantly to the active form of alphaVbeta3 integrin. Examination by confocal microscopy found that LM609 antibodies accumulate in alphaVbeta3 and strong colocalization with PDI in focal adhesion in response to adhesion. This observation is consistent with previous data showing that PDI interacts directly with the active conformer of alphaVbeta3 integrin (5). Our findings also confirm that PDI is constitutively secreted from endothelial cells, as noted previously (11). Induction of the high-affinity state of alphaVbeta3 involves a thiol-dependent step associated with integrin activation. When endothelial cells adhere to extracellular matrix proteins, free thiol groups are generated within alphaVbeta3 and PDI molecules. Several studies have reported that free thiol groups contribute to integrin activation (28,29).

Our findings confirm that the activation of alphaVbeta3 integrins causes the release of PDI from endothelial cells. The secreted PDI rebinds to the integrin receptor and maintains it in an active state. In addition, the secretion of PDI from endothelial cells requires a functionally-active alphaVbeta3 integrin.

## 6. ACKNOWLEDGEMENTS

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**Abbreviations:** BSA, bovine serum albumin; DENV, dengue virus; DMEM, Dulbecco's modified Eagle medium; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); FAK, focal adhesion kinase; FBS, fetal bovine serum; GRO-alpha, growth-related oncogene-alpha; GSH, reduced glutathione; HAT, hypoxanthine, aminopterin, thymidine; HUVEC, human umbilical vein endothelial cells; MPB, *N*-(3-Maleimidopropionyl)biocytin; PCMBS, p-chloromercuribenzenesulfonic acid; PDI, protein disulfide isomerase; RGD, Arg-Gly-Asp amino acid sequence; RDE**,** Arg-Gly- Glu amino acid sequence; Src, sarcoma-family kinases

**Key words:** PDI, Thiol Groups, Integrins, Adhesion, Endothelial Cells

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