Chronic exposure to fibrin and fibrinogen differentially regulates intracellular Ca\(^{2+}\) in human pulmonary arterial smooth muscle and endothelial cells

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Acute pulmonary embolism occurs in more than half a million people a year in the United States. Chronic thromboembolic pulmonary hypertension (CTEPH) develops in ~4% of these patients due to unresolved thromboemboli. CTEPH is thus a relatively common, progressive, and potentially fatal disease. One currently proposed theory for the poor resolution advocates that modification of fibrinogen in CTEPH patients causes resistance of emboli to fibrinolysis.

The current study investigated the regulation of cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{cyt}\)) central to the control of cell migration, proliferation, and contraction, by chronic exposure of pulmonary artery smooth muscle (PASMC) and endothelial (PAEC) cells to fibrinogen and fibrin. Basal [Ca\(^{2+}\)]\(_{cyt}\) was substantially elevated in PAEC after culture on fibrinogen, fibrin, and thrombin in PASMC on fibrinogen and fibrin. In PAEC, fibrinogen significantly decreased the peak [Ca\(^{2+}\)]\(_{cyt}\) transient (P <0.001) without a change in the transient peak width (at 50% of the peak height).

This response was independent of effects on the proteinase-activated receptor (PAR)-1 activity on thrombin, an activator of PAR, significantly reduced the peak agonist-induced Ca\(^{2+}\) release in PAEC, but increased it in PASMC. The recovery rate of the agonist-induced [Ca\(^{2+}\)]\(_{cyt}\) transients decelerated in PASMC chronically exposed to fibrin; a small increase of the peak Ca\(^{2+}\) was also observed. Substantial augmentation of PASMC (but not PAEC) proliferation was observed in response to chronic fibrin exposure. In conclusion, chronic exposure to fibrinogen, fibrin, and thrombin caused differential changes in [Ca\(^{2+}\)]\(_{cyt}\) in PAEC and PASMC. Such changes in [Ca\(^{2+}\)]\(_{cyt}\) may contribute to vascular changes in patients who have CTEPH where the pulmonary vasculature is persistently exposed to thromboemboli.

Thrombin; pulmonary vascular remodeling; calcium regulation

DESPITE VAST INVESTIGATION into the mechanisms of pulmonary hypertension, one particular disease that is rarely considered is chronic thromboembolic pulmonary hypertension (CTEPH). CTEPH is a very serious complication of an acute pulmonary embolism, occurring in more than 4% of patients with pulmonary embolism (or ~20,000 cases per year). It has been suggested that the prevalence and incidence of CTEPH are deemed to be grossly underestimated. Although the clinical and hemodynamic features of the disease are well documented, there is a distinct deficit of pathophysiological studies. To develop effective therapeutic and diagnostic approaches, a full understanding of the cellular and molecular pathophysiology associated with CTEPH is a necessity.

Rationale for why the pulmonary embolism is resistant to conventional, anticoagulant, and vasodilator treatment is currently unknown. One promising theory advocates that the fibrin in CTEPH patients is modified and resistant to fibrinolysis via adaptations to the β-chain in the NH\(_2\) terminus (15). An abnormally high amount of γ-Asn-52 disialylated renders the clot resistant to plasmin and unusually transparent in some patients (17). It is possible that this resistance may originate from mutations within the fibrinogen gene. Recently, a substitution of threonine with alanine at position 312 (Thr312Ala) in fibrinogen was shown to correspond with significant genotype and allele frequencies between CTEPH and control subjects. This polymorphism affects the fibrinogen α-α chain cross-linkage and increases resistance to thrombolysis (23).

Thrombin, a serine protease that catalyzes the conversion of fibrinogen to fibrin, is known to have potent effects on endothelial cells leading to endothelial barrier dysfunction due to mobilization of Ca\(^{2+}\) and rearrangement of the cytoskeleton (6). Furthermore, thrombin has been extensively investigated for its role in atherosclerosis (10), being a potent mitogen for smooth muscle cells of the vasculature (5). By binding to protease-activated receptors expressed on smooth muscle cells (PAR-1, -3, and -4), thrombin can modulate Ca\(^{2+}\) signaling and regulates cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)) in turn increasing cell proliferation and migration (1, 14). At physiological concentrations, thrombin-induced increases in plasminogen activator-inhibitor (PA-I) may protect clots from premature lysis (8). A decrease in plasma concentration of thrombomodulin (another plasma membrane thrombin receptor on endothelial cells) may provide one plausible contributory factor for the decreased physiological anticoagulant activity and fibrinolytic function in CTEPH patients (21).

Due to the decreased fibrinolysis/thrombolysis in these patients, the vascular wall is subjected to a prolonged exposure to components of the final common pathway of the coagulation cascade. The long-term effects of fibrinogen and fibrin on human PASMC and PAEC have not been examined; therefore, this study investigates the effect of fibrinogen and fibrin on these cells focusing on cellular mechanisms known to contribute to intracellular Ca\(^{2+}\) signaling and vascular remodeling.

MATERIALS AND METHODS

Fibrinogen purification. Human plasma fibrinogen was either bought from Calbiochem (San Diego, CA) or purified from blood samples from normal subject. Approval was granted by the UCSD Institutional Review Board. Briefly, blood was collected in Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and immediately placed...
on ice in a one-tenth volume of buffered sodium citrate (3.8%) before separation of the plasma by centrifugation at 2,000 g for 10 min at 4°C. Fibrinogen was purified using an ethanol precipitation methodology (7). The concentration, determined by UV spectroscopy, and quality of the fibrinogen, assessed by its clotability and purity, was routinely assessed as previously described by Morris et al. (15). The fibrinogen purified for experiments was typically more than 90% pure and contained more than 90% clottable protein.

**Cell preparation and culture.** Sterile 25-mm circular glass coverslips were coated in six-well culture dishes. Coverslips were either coated with fibrinogen, 2 ml of two concentrations, 4 and 40 μg/ml diluted in PBS, or PBS alone and incubated for a minimum of 3 h at room temperature. All coverslips were blocked for 1 h with PBS containing 1 mg/ml BSA. For the fibrin-coated coverslips, 0.2 U/ml thrombin was added to the blocking solution. This created six different experimental conditions: control, 4 and 40 μg/ml fibrinogen, thrombin, and 4 and 40 μg/ml fibrin. Where Phe-Pro-Arg-chloromethylketone (PPACK) was used, 0.01 μg/ml PPACK was added for 20 min after blocking. Successful coating of the coverslips was confirmed using a goat polyclonal thrombin (K20) antibody (Santa Cruz, CA), a polyclonal rabbit antibody that reacts strongly with fibrinogen, and a murine monoclonal antibody (IgG1) raised against a synthetic peptide identical to the first seven residues of the β-chain of human fibrinogen obtained as previously described (18). Each was detected with secondary antibodies conjugated to HRP (1:5,000) and visualized using enhanced chemiluminescence on photographic film. Cells, human PASMC, or PAEC (Lonza, Walkersville, MA) were plated on the coated coverslips for 72 h at 37°C in the smooth muscle growth medium (SmGM) composed of smooth muscle basal medium (SmBM) supplemented with 5% fetal bovine serum, 0.5 ng/ml human epidermal growth factor, 2 ng/ml human fibroblast growth factor, and 5 μg/ml insulin or endothelial growth medium (EGM) composed of endothelial basal medium (EBM) supplemented with 2% FBS, human epidermal growth factor, human fibroblast growth factor, and insulin.

**Clot formation.** Irreversible thrombin inhibitor PPACK was preincubated with thrombin for 15 min at room temperature, and the mixture was added to fibrinogen and monitored for clot formation. Clot formation was determined by an increase in optical density measured on a spectrophotometer. The thrombin concentration in the preincubation mixture was 2 U/ml and 0.2 U/ml in the clot mixture. The fibrinogen concentration in the clot mixture was 1.4 mg/ml. The clot mixture also contained 10 mM CaCl2.

**Measurement of [Ca2+]cyt.** Cells were used at passage 6 or lower and were ~60% confluent at 72 h. Cells on 25-mm circular coverslips were placed in a recording cell chamber on the stage of an inverted Nikon microscope (Eclipse/TE 200) with the TE-FM epifluorescence attachment. Cells were loaded with the membrane-permeable acetoxymethyl ester form of fura-2 (fura-2-AM, 3 μM) for 30 min in the dark at room temperature (22-24°C). The fura-2-AM-loaded cells were then superfused with modified Krebs solution (MKS) for 30 min at 32°C to wash away extracellular dye and to permit intracellular cleavage of fura-2-AM to active fura-2 by cytoplasmic esterases. Fura-2 fluorescence from arbitrarily chosen peripheral cytoplasmic regions from each cell and background fluorescence were monitored continuously using a fluorescence microscopy system (Intracellular Imaging). Data were collected at 1-s intervals using Nikon UV-Fluor objectives (excitation 340 and 380 nm with xenon lamp, emission 520 nm) using ×40 magnification.

**Proliferation and [3H]thymidine uptake measurement.** Cells, human PASMC, or PAEC were plated at 1 × 105 cells/well on the coated wells, as described above, for 24 h in SMGM or EGM, respectively. They were then serum starved for 24 h (SMBM or EBM) before replacement with basal media containing 5% FBS and 1 μCi [3H]thymidine for 24 h. Controls were assessed in parallel without serum stimulation. Wells were washed with cold PBS and twice with cold 7.5% TCA, and before the addition of 0.5 M NaOH to dissolve the cell walls. Liquid scintillation counting was used to determine the extent of DNA synthesis from the [3H]thymidine incorporation. Proliferation is expressed as the serum-stimulated growth as a percentage relative to the control (no serum). Each n represents one experiment that is an average of two to four paired wells of cells. All conditions were assessed in parallel from the same originating flask of cells per experiment; six individual experiments were carried out.

**Solution and reagents.** Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN) provided at a concentration of 3 mg/ml with an activity of 3,133 NIH U/ml. A 1/100 stock solution was prepared by dilution of the original preparation in 0.1% PEG-3350 containing 0.2 M NaCl and stored in 50-μl aliquots at −80°C. MKS contained (in mM): 138 NaCl, 4.7 KCl, 1.2 NaH2PO4, 1.2 MgSO4, 10 HEPES, 18 CaCl2, 10 glucose, and pH adjusted to 7.4 using NaOH. For Ca2+-free MKS, CaCl2 was replaced with equimolar MgCl2, and 1 mM EGTA was added to chelate residual Ca2+. The pH values of all solutions were checked after the addition of drugs and were readjusted to 7.4. Fura-2-AM was purchased from Invitrogen (Carlsbad, CA) and dissolved in DMSO for use on the same day. PAR-1 agonist TFLLRN and PAR-3 agonist SFNGG-P-NH2 were purchased from AnaSpec and diluted in MKS to a working concentration of 10 μM.

**Statistical analysis.** Data are expressed as means ± SE. Statistical analysis was performed by Student's t-test. Data were considered to be statistically significant when P < 0.05.

**RESULTS**

Antibody detection of fibrin and fibrinogen confirmed the specific coating of coverslips. All the respective controls were performed (data not shown). Thrombin was detected on the thrombin-only-coated coverslip; in addition, some residual thrombin was detected on the fibrin-coated coverslips. The residual thrombin may resemble a physiological, postclotting thrombin (bound thrombin) as identified by Sunagawa et al. (22).

Fibrinogen and fibrin increase resting [Ca2+]cyt in PAEC. The effects of 72-h culture on a fibrinogen-coated surface (4 and 40 μg/ml) compared with control (no coating) on agonist-induced [Ca2+]cyt transients were assessed in PAEC and PASMC. The resting or basal [Ca2+]cyt in PAEC after culture on fibrinogen and fibrin was significantly elevated (Fig. 1A). Consistent with the effects in PAEC, fibrinogen and fibrin also caused a significant elevation of resting [Ca2+]cyt in PASMC (Fig. 1B). Interestingly, thrombin-coated surfaces caused a significant increase in resting [Ca2+]cyt only in PAEC (Fig. 1, A and B).

Fibrinogen selectively decreases Ca2+ release in PAEC. Transient elevations of [Ca2+]cyt evoked by acute exposure to agonist thrombin (5 nM) were significantly reduced in PAEC cultured on fibrinogen (P < 0.01) (Fig. 2, A and Ba) with no significant effect on the time course of the transient (Fig. 2, Bb and Bc). A similar trend (but not a statistically significant effect) was observed after culture on fibrin-coated surfaces. In contrast, only the higher concentration of fibrinogen (40 μg/ml) had a significant affect on the acute thrombin-mediated increase in [Ca2+]cyt in PASMC (Fig. 3, A and Bb), decreasing the peak but concurrently significantly slowing the recovery of the transient increase in [Ca2+]cyt (Fig. 3, Bb and Bc). These results provide an interesting observation that chronic exposure to fibrinogen causes a rather different effect on transient changes in [Ca2+]cyt induced by acute treatment with 5 nM thrombin between PAEC and PASMC.

Fibrin increases Ca2+ release in PASMC. Fibrin monomers were created by cleavage of fibrinopeptidases A and B from the
NH₂-terminal ends of fibrinogen (coated surfaces) with thrombin (0.2 U/ml). Ca²⁺ was not present, thus preventing the formation of opaque clots due to cross-linking of the polymers to allow visualization of the cells on an inverted microscope for analysis. Again, agonist-induced [Ca²⁺]ᵣᵣ transients were assessed in PAEC and PASMC cultured for 72 h on a fibrin-coated surface (4 and 40 μg/ml fibrinogen) compared with control (no coating or thrombin-only treated surfaces).

In PAEC, chronic exposure to a fibrin surface caused a small (but not statistically significant) decrease in the thrombin-induced [Ca²⁺]ᵣᵣ transient (Fig. 2, A and Bb), with no substantial effect on the time course (Fig. 2, Bb and Bc). Conversely, acute thrombin-induced transient increases in [Ca²⁺]ᵣᵣ in PASMC were enhanced by chronic exposure to fibrin (Fig. 3, A and Ba), and this was associated with a significant slowing of the recovery of the [Ca²⁺]ᵣᵣ transient to resting levels (Fig. 3, Bb and Bc). These data indicate that fibrin raises basal [Ca²⁺]ᵣᵣ in PAEC with little effect on agonist-mediated increases in [Ca²⁺]ᵣᵣ. On the other hand, fibrin augments the agonist-mediated increases [Ca²⁺]ᵣᵣ in PASMC (by decelerating the recovery of thrombin-induced [Ca²⁺]ᵣᵣ transients in PASMC).

Chronic exposure to thrombin desensitizes receptors in PAEC. Although the acute effects of thrombin are being assessed in this study and used as a parameter to determine the effects of chronic exposure of PAEC and PASMC to fibrinogen and fibrin, we also examined whether chronic exposure of cells to thrombin (5 nM, for 72 h) affected the transient increase in [Ca²⁺]ᵣᵣ.
FIBRIN REGULATES INTRACELLULAR Ca²⁺ IN PASMC AND PAEC

[Ca²⁺]cyt induced by acute thrombin treatment. As shown in Fig. 1, the resting [Ca²⁺]cyt was significantly (P < 0.001) higher in PAEC (1.56 ± 0.02 vs. 1.76 ± 0.02 F₃₄₀/F₃₈₀, n = 257 and 114 for control and thrombin-treated cells, respectively), although not significantly altered in PASMC. Interestingly, inhibition of thrombin by PPACK, an irreversible selective inhibitor of thrombin acting by cleaving the active site, significantly decreased the basal [Ca²⁺]cyt compared with thrombin coating without the inhibitor (P < 0.001, Fig. 4). The amplitude of acute thrombin-induced increase in [Ca²⁺]cyt was, however, significantly decreased in PAEC (P < 0.001, Fig. 2, A and Ba) without significant effect on the kinetics of [Ca²⁺]cyt transient recovery (Fig. 2, Bb and Bc). In contrast, chronic exposure of PASMC to thrombin actually enhanced the amplitude of acute thrombin-induced [Ca²⁺]cyt transients (Fig. 3, A and Ba) with no concurrent effect on the kinetics of transient recovery (Fig. 3, Bb and Bc). These observations indicate that chronic exposure to thrombin may desensitize the thrombin receptors in PAEC, but not in PASMC. Interestingly, inhibition of thrombin using PPACK (0.1 g/ml) did not significantly alter the agonist-mediated Ca²⁺ transients compared with the thrombin in the absence of the inhibitor (Fig. 4C). PPACK at concentrations of 0.01 and 0.1 g/ml was sufficient to inhibit clot formation assessed by changes in turbidity (Fig. 4A).

Active thrombin mixed with fibrinogen forms a clot that is associated with an increase in optical density (OD) as determined by a spectrophotometer. This suggests that the chronic thrombin-mediated effects do not require active thrombin and [Ca²⁺]cyt transients in PASMC and PAEC.

Fig. 3. Fibrin treatment enhances thrombin-induced increase in [Ca²⁺]cyt in PASMC. A: representative [Ca²⁺]cyt traces indicating thrombin-mediated increases in [Ca²⁺]cyt in control (a) and when cultured on a surface of 4 μg/ml (b) or 40 μg/ml (c) FGN for 72 h or 4 μg/ml (d) or 40 μg/ml (e) fibrin, and 0.2 U/ml thrombin (f) for 72 h. B: analysis of the thrombin-induced changes in [Ca²⁺]cyt, including the peak increase in [Ca²⁺]cyt (a), the time constant (t_decay) for the recovery to basal [Ca²⁺]cyt, levels measured in seconds (b), and the width of the [Ca²⁺]cyt transient measured at 50% of the maximal increase in [Ca²⁺]cyt (c). *P < 0.05, **P < 0.001 vs. control; n = 30–250 cells and 3–5 experiments per condition.

Fig. 4. Phe-Pro-Arg-chloromethylketone (PPACK) decreases chronic thrombin-mediated changes in basal [Ca²⁺]cyt, but not acute [Ca²⁺]cyt transients in PASMC. A: concentration-dependent effects of PPACK on clot formation determined by turbidity-dependent changes in optical density (O. D.). B: basal [Ca²⁺]cyt, in PASMC after the cells were cultured for 72 h on thrombin or thrombin and PPACK-coated surfaces. C: analysis of the thrombin-induced changes in [Ca²⁺]cyt, including the peak increase in [Ca²⁺]cyt (left), the width of the [Ca²⁺]cyt transient measured at 50% of the maximal increase in [Ca²⁺]cyt (middle), and the area under the curve (right). *P < 0.05, ***P < 0.001 vs. control.
are thus unlikely due to thrombin’s actions on thrombin receptors such as PAR-1, PAR-3, or PAR-4.

PAR-1 agonist-mediated changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) are unchanged by fibrinogen. To investigate potential changes in PAR-1 activity, the effect of the PAR-1-specific agonist TFLLRN was assessed. In PAEC, TFLLRN mediated a significant Ca\(^{2+}\) transient that was not significantly changed between cells cultured on control and fibrinogen-coated surfaces (Fig. 5, A and B). Interestingly, while subsequent application of TFLLRN only induced a very small Ca\(^{2+}\) release, indicative of irreversible cleavage of the PAR-1 receptor by TFLLRN, subsequent exposure of the cells to thrombin still caused a significant Ca\(^{2+}\) transient (Fig. 5, A and B). This Ca\(^{2+}\) transient was significantly reduced in PAEC cultured on the fibrinogen surface compared with control, indicative of a PAR-1-independent mechanism for fibrinogen-induced decrease in thrombin-mediated Ca\(^{2+}\) release. In PASMC, PAR-1-induced Ca\(^{2+}\) release only accounted for a very small percentage of the total thrombin-mediated effect (Fig. 6).

2-APB and lanthanum reduce chronic elevation of basal Ca\(^{2+}\). 2-APB is a reliable inhibitor of store-operated Ca\(^{2+}\) influx (SOC) and inconsistently a blocker of IP\(_3\)-mediated Ca\(^{2+}\) release (4). 2-APB (80 \(\mu\)M) prevented the chronic fibrinogen-induced elevation of basal \([\text{Ca}^{2+}]_{\text{cyt}}\) (Fig. 7A). While 2-APB significantly blocked CPA-induced elevations of \([\text{Ca}^{2+}]_{\text{cyt}}\) and completely prevented CCE (Fig. 7B), it, although significantly (\(P < 0.05\)), only reduced the thrombin-mediated rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) by a small amount (Fig. 7C). This suggests that the acute thrombin-mediated elevations of \([\text{Ca}^{2+}]_{\text{cyt}}\) are not predominantly due to Ca\(^{2+}\) influx via SOC. Lanthanum (La\(^{3+}\); 50 \(\mu\)M) mimicked the effects of 2-APB preventing the
Fibrinogen-dependent chronic elevation of basal \([\text{Ca}^{2+}]_{\text{cyt}}\) (Fig. 7A) and significantly inhibiting the acute thrombin-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) peak (Fig. 7B).

**Fibrin increases proliferation only in PASMC.** Proliferation of human PASMC and PAEC was investigated using a \(^3\text{H}\)thymidine uptake proliferation assay. In PAEC, no significant changes in cell proliferation were observed for any of the culture conditions compared with control (data not shown). In PASMC, however, serum-stimulated proliferation, determined by \(^3\text{H}\)thymidine incorporation, was increased when cells were cultured on the fibrinogen (40 \(\mu\)g/ml) or thrombin (5 nM) surfaces (from 100% in control to 133.9 ± 7.6% and 143.5 ± 15.8%, respectively, \(n = 6\)) (Fig. 8). Fibrin (40 \(\mu\)g/ml) did substantially increase the proliferation rate; however, the serum-stimulated expansion was not significant. Data are shown as raw data (Fig. 8A) in the presence and absence of serum and as serum-stimulated proliferation (calculated as “with serum” minus “without serum” per experiment) expressed as a percentage of the control condition (Fig. 8B). These data indicate that chronic exposure of PASMC to fibrinogen, fibrin, and thrombin substantially enhances PASMC proliferation. These observations further suggest that accumulation or penetration of circulating fibrinogen, fibrin, and thrombin into the pulmonary vascular media, for example, when endothelial injury occurs, may serve as a sustained stimulation for smooth muscle cell growth and proliferation, and ultimately causing pulmonary vascular remodeling.

**DISCUSSION**

This study considers an important functional significance of the prolonged exposure of the pulmonary arteries to the final common pathway of the coagulation cascade, comprising the effects of fibrinogen, fibrin, and thrombin on the cellular responses of human PAEC and PASMC. Studies into the pathophysiological mechanisms of CTEPH are sparse. Recent studies have proposed a role for staphylococci infection in the poor thrombus resolution and evolution of CTEPH (3) and increased plasma lipoprotein a levels (9). A high resting fibrinogen-dependent chronic elevation of basal \([\text{Ca}^{2+}]_{\text{cyt}}\) (Fig. 7A) and significantly inhibiting the acute thrombin-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) peak (Fig. 7B).
Fibrinogen and fibrin elevate resting Ca\(^{2+}\) in PAEC. Recent findings that a Thr312Ala polymorphism alters the cross-linking of fibrinogen increasing the resistance to thrombolysis is prevalent in CTEPH patients (23); this provides a rationale for the persistence of the thrombus enabling the conversion to a fibrous structure permanently occluding the pulmonary artery. Although fibrinogen is rapidly converted to fibrin by thrombin, our data suggest that exposure of the endothelial cell layer to both fibrinogen and fibrin causes a significant increase in the basal [Ca\(^{2+}\)]\(_{cyt}\). This chronic elevation of basal [Ca\(^{2+}\)]\(_{cyt}\) is sensitive to 2-APB (a reliable SOC inhibitor and IP\(_3\) receptor blocker) (4) and La\(^{3+}\) [a SOC inhibitor and calcium sensing receptor (CaSR) agonist](2, 24). SOC expression or activity is therefore potentially upregulated by fibrinogen, fibrin, and thrombin-coated surfaces in PAEC. The decreased Ca\(^{2+}\) release when these cells were stimulated by thrombin is not surprising given that the potential for further increases in [Ca\(^{2+}\)]\(_{cyt}\) are decreased due to the already elevated basal [Ca\(^{2+}\)]\(_{cyt}\).

Fibrinogen decreases in thrombin-mediated elevation of [Ca\(^{2+}\)]\(_{cyt}\) are PAR-1 independent. PAEC are known to express functional PAR (11, 20). The PAR-1-specific agonist TFLLRN (10 \(\mu\)M) induced a significant transient elevation of [Ca\(^{2+}\)]\(_{cyt}\) in PAEC that remained unaltered in PAEC cultures on fibrinogen surfaces. Interestingly, the significant decrease in the subsequent thrombin-mediated effect was still observed reflecting the decreased Ca\(^{2+}\) transient on PAEC chronically exposed to fibrinogen. The fibrinogen-mediated effects therefore seem to be independent of thrombin’s activity at PAR-1 in PAEC. A similar response was observed in PASMC, however. The PAR-1-specific effect on [Ca\(^{2+}\)]\(_{cyt}\) was very small. These data indicated that the decreased agonist-induced acute effect on [Ca\(^{2+}\)]\(_{cyt}\) by fibrinogen was PAR-1 independent in PAEC and PASMC. It remains plausible that PAR-1 could be involved, but indirectly, via the allosteric actions of PAR-3 in regulating PAR-1 (13). Although we observed no significant changes in [Ca\(^{2+}\)]\(_{cyt}\) by PAR-3-selective agonist in PAEC (data not shown), supportive of the notion that PAR-3 is a nonsignaling receptor, changes in PAR-3 expression may be involved in regulating PAR-1-mediated changes in [Ca\(^{2+}\)]\(_{cyt}\) (13).

Fibrin stimulates intracellular Ca\(^{2+}\) release and proliferation in PASMC. The resistance of the fibrin to fibrinolysis and excessive \(\beta\)- and \(\gamma\)-chain sialylation has been documented for CTEPH (15, 17). In this study, it was observed that increased [Ca\(^{2+}\)]\(_{cyt}\) and augmented PASMC proliferation occurred in response to chronic fibrin exposure alongside a significant increase in the resting [Ca\(^{2+}\)]\(_{cyt}\) levels when PASMC were chronically exposed to either fibrinogen or fibrin. Fibrin also sensitized PASMC to acute thrombin exposure, increasing the recovery time of the Ca\(^{2+}\) transient. The persistence of fibrin in the thromboemboli is thought to be the key parameter for the formation of the “vascular scar” in the development of CTEPH, and, as our data indicates, may be a potential stimulus for pathophysiological changes in the pulmonary vasculature.

Thrombin desensitized receptors/signaling in PAEC. The prolonged exposure to thrombin decreased the thrombin-mediated [Ca\(^{2+}\)]\(_{cyt}\) transients supporting previous observations that >60-min preexposure to thrombin abolished increases in [Ca\(^{2+}\)]\(_{cyt}\) (6). While the studies by Ellis and colleagues (6) provided evidence for a desensitization of endothelial plasma membrane PAR-1 receptors, the data in the current manuscript suggest a thrombin-dependent, PAR-1-independent change in [Ca\(^{2+}\)]\(_{cyt}\). A complete inhibition of the Ca\(^{2+}\) release was not observed after 72 h; however, inhibition of active thrombin by PPACK did not significantly affect the observed acute changes in [Ca\(^{2+}\)]\(_{cyt}\). It is worth noting that PPACK did significantly reduce basal [Ca\(^{2+}\)]\(_{cyt}\) compared with thrombin alone, suggesting that active thrombin is not required for the changes in acute agonist-mediated effects on [Ca\(^{2+}\)]\(_{cyt}\), but does influence the chronic effect of the thrombin-coated surfaces. In PASMC, we observed a significant increase in intracellular Ca\(^{2+}\) release in concert with stimulated proliferation corroborating previously published data in aortic smooth muscle cells (1) where a prolonged exposure to thrombin was necessary to stimulate increased mitogenic activity via an increase in growth factor release and receptor expression.

In conclusion, we observed an elevation of basal [Ca\(^{2+}\)]\(_{cyt}\) in PAEC when chronically exposed to either fibrinogen or fibrin, which is likely to lead to a dysfunctional endothelial cell layer. These decreased acute effects of thrombin occur via a PAR-1-independent mechanism. Exposure of PASMC to fibrin, which enhances the thrombin-mediated increases in [Ca\(^{2+}\)]\(_{cyt}\), may augment contraction, migration, and proliferation of these cells. Stimulation of these processes will potentially lead to pulmonary vascular remodeling and contribute to the persistence of pulmonary hypertension in CTEPH patients.

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