\(\alpha_\beta_3\) Integrin Induces Tyrosine Phosphorylation–Dependent \(\text{Ca}^{2+}\) Influx in Pulmonary Endothelial Cells

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Abstract—The endothelial \(\alpha_\beta_3\) integrin occurs luminally, where its ligation by soluble agents may induce inflammatory signaling. We tested this hypothesis in bovine pulmonary artery endothelial cell monolayers with the use of vitronectin and cross-linking antibodies to ligate and aggregate the integrin. We quantified the endothelial cytosolic \(\text{Ca}^{2+}\) concentration ([Ca\(^{2+}\)]) according to the Fura 2 ratio imaging method in single cells of confluent monolayers. At baseline, endothelial [Ca\(^{2+}\)] levels remained steady at 86 nmol/L for >20 minutes. Cross-linking of the \(\alpha_\beta_3\) integrin through the sequential exposure of monolayers to anti-\(\alpha_\beta_3\) monoclonal antibody LM609 and secondary IgG resulted in a [Ca\(^{2+}\)] increase of 100% above baseline. This increase commenced in <0.5 minute, peaked in <2 minutes, and decayed to baseline in \(\approx 5\) minutes. Similar responses occurred after the addition of vitronectin (400 \(\mu\)g/mL). In contrast, external \(\text{Ca}^{2+}\) depletion blunted the cross-linking–induced [Ca\(^{2+}\)] increase by 60%, a response that was completely inhibited when the monolayers were also pretreated with thapsigargin. Thus, the [Ca\(^{2+}\)] increase was attributable in part to the release of Ca\(^{2+}\) from endosomal stores but mostly to Ca\(^{2+}\) influx across the plasma membrane. Induced aggregation of the \(\alpha_\beta_3\) integrin enhanced tyrosine phosphorylation of phospholipase C-\(\gamma_1\) and increased the accumulation of inositol-1,4,5-trisphosphate. Genistein, a broad-spectrum tyrosine kinase inhibitor, abrogated both of these effects, as well as the \(\alpha_\beta_3\)-induced [Ca\(^{2+}\)] increases. We conclude that aggregation of the endothelial \(\alpha_\beta_3\) integrin induces a rapid tyrosine phosphorylation–dependent increase in [Ca\(^{2+}\)]. This response may subserve the inflammatory role of \(\alpha_\beta_3\) integrin in blood vessels. (Circ Res. 2000;86:456–462.)

Key Words: integrins ■ endothelium ■ cells ■ vitronectin ■ phospholipases

The endothelial \(\alpha_\beta_3\) integrin is important in angiogenesis, vasculogenesis, and vascular cell survival.\(^1\)\(^,\)\(^2\) These are matrix-dependent functions that probably in large part involve integrins expressed on the matrix-facing aspect of the endothelial cell. However, the endothelial \(\alpha_\beta_3\) integrin also exists on the luminal, blood-facing aspect,\(^3\) where it is likely to function as a receptor for blood-borne ligands. Our finding that the luminal \(\alpha_\beta_3\) integrin increases lung capillary permeability\(^4\) indicates that the luminal integrin may subserve inflammatory endothelial responses.

Rapid mobilization of endothelial Ca\(^{2+}\) is often characteristic of inflammatory processes. Increases in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) trigger vascular responses such as an increase in capillary permeability,\(^5\) the secretion of inflammatory cytokines,\(^6\) and the induction of gene transcription.\(^7\) However, \(\alpha_\beta_3\)-induced [Ca\(^{2+}\)] responses have not been determined in stable endothelial cells.\(^8\) In endothelial cells allowed to spread on immobilized \(\alpha_\beta_3\) ligands, [Ca\(^{2+}\)] increases occur gradually and reach a peak at \(\approx 30\) minutes.\(^8\) These slow [Ca\(^{2+}\)] increases do not account for rapid endothelial responses such as barrier deterioration that occur in <1 minute.\(^4\) Soluble \(\alpha_\beta_3\) ligands decrease [Ca\(^{2+}\)], in osteoclasts\(^9\) and myocytes,\(^10\) a response that if true for endothelial cells, may argue in favor of a barrier-protective, not a barrier-deteriorating, effect.

Receptor-mediated endothelial [Ca\(^{2+}\)] increases may result from a sequence in which phospholipase C-\(\gamma\) (PLC-\(\gamma\)) activation leads to the release of inositol-1,4,5-trisphosphate (InsP\(_3\)), store release of Ca\(^{2+}\), and entry of external Ca\(^{2+}\).\(^11\) These well reported mechanisms apply to several receptors that activate PLC-\(\gamma\) through tyrosine phosphorylation.\(^12\) Although the \(\alpha_\beta_3\) integrin may fall in this category,\(^12\) mechanisms remain confusing because protein tyrosine phosphorylation may itself be Ca\(^{2+}\) enhanced.\(^13,\)\(^14\) However, it is also possible that [Ca\(^{2+}\)], elevation inhibits the phosphorylation.\(^15,\)\(^16\) We considered these possibilities in the context of the \(\alpha_\beta_3\) integrin.

Multivalent vitronectin aggregates the \(\alpha_\beta_3\) integrin and enhances protein tyrosine phosphorylation.\(^17\) Complement activation increases plasma levels of the vitronectin-containing complement complexes.\(^18\) Such complexes, as well as other vitronectin-binding inflammatory factors, such...
as bacteria, viruses, 19-21 and the multivalent thrombin-antithrombin complex, 22 may ligate the luminal αβ3 integrin during inflammatory and thrombotic states. Ensuing αβ3 integrin-mediated enhanced protein tyrosine phosphorylation may then initiate inflammatory responses.

In the present study, we used pulmonary endothelial monolayers to model responses relevant to our previously reported findings in lung capillaries. 4 Our main aim was to determine whether αβ3 ligation sufficiently aggregates the integrin to rapidly increase endothelial [Ca2+]i. Our strategy was to aggregate the integrin with the use of cross-linking antibodies or multimeric vitronectin. Our findings indicate that both caused tyrosine phosphorylation–induced [Ca2+]i increases that unexpectedly initiated at the cell periphery. We discuss the implications of these findings in relation to the permeability-enhancing effect of the integrin in capillaries.

Materials and Methods

Cells, Reagents, and Antibodies

Bovine pulmonary artery endothelial cells (BPAECs) (American Type Culture Collection) were grown to confluence in DMEM, and immunofluorescent staining for factor VIII antigen was confirmed. EGTA, genistein, and thapsigargin were purchased from Sigma Chemical Co. MAPTAM (1,2-bis-5-methyl-amino-phenoxylethane-N,N,N’-tetra-acetoxyethylate) was purchased from Calbiochem. Secondary antibody (Ab) and donkey anti-mouse IgG were provided by D. Cheresh (Scripps Clinic and Research Foundation, La Jolla, Calif). Multimeric vitronectin was purified from human plasma as described previously. 17

Cross-Linking Protocols

The αβ3 integrin was cross-linked through the exposure of confluent BPAEC monolayers first to LM609 (200 μg/mL, 30 minutes, 4°C) and then to donkey anti-mouse IgG (30 μg/mL, 5 minutes, 37°C). For Ca2+-free conditions, the secondary IgG was added in Ca2+-free buffer containing 0.5 mM/LETTA. For the immunofluorescent detection of αβ3 aggregation, BPAECs were lightly fixed (3.7% paraformaldehyde, 20 minutes, 22°C) and permeabilized (0.5% Triton X-100, 2 minutes) according to Lawson et al. 23 Then, αβ3 was cross-linked with Cy3-conjugated LM609 as the primary Ab. Immunofluorescence was detected with confocal (Insight +; Meridian Instrument Co) and conventional (Olympus LHS0A) fluorescence microscopy.

Ca2+ Imaging of Single Endothelial Cells

Our methods for Ca2+ imaging according to the Fura 2 ratio method were described previously. 24 BPAEC monolayers were Fura 2 loaded through the addition of Fura 2-AM (5 μmol/L, 30 minutes, 20°C) and then maintained at 37°C during digital imaging. [Ca2+]i was determined in a 2-μm window placed over 340/380 ratio images of single cells, based on appropriate calibrations and a Fura 2Ca2+/K0 value of 224 nmol/L. 25

Immunoblotting and Immunoprecipitation

Cells were lysed in ice-cold lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris base, 2 mmol/L EDTA, 50 mmol/L NaF, 0.1% SDS, 1% NP-40, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate, the phosphatase inhibitor, pH 7.5). Lysates were cleared through centrifugation (14,000 rpm, 15 minutes), and protein concentrations were determined according to the DC Protein Assay (Bio-Rad). Anti-phosphotyrosine immunoblotting was performed as described previously. 17 Cell lysates containing equal amounts of protein were electrophoresed onto 10% SDS-polyacrylamide gels under reducing conditions. After electrophoretic transfer to nitrocellulose, phosphotyrosyl-containing proteins were detected with affinity-purified anti-phosphotyrosine IgG that was previously derivatized with sulfosuccinimidylbiotin, followed by the addition of streptavidin-horseradish peroxidase. Blots were developed with the use of enhanced chemiluminescence. Immunoprecipitation was performed as described previously. 17

Statistical Analysis

All values are given as mean±SEM. Differences between groups were tested with the paired t test for 2 groups and the Newman-Keuls test for >2 groups. Statistical significance was accepted at P<0.05.

Results

Immunofluorescence Studies of Clustered αβ3 in BPAEC Monolayers

To determine [Ca2+]i responses to αβ3 clustering, we used cross-linking Abs as well as vitronectin. The binding affinity of vitronectin to the αβ3 integrin is Ca2+-dependent. 26 The advantage of cross-linking is that the binding affinity of the anti-αβ3, mAb LM609 is both αβ3 specific and Ca2+-independent. 27 Accordingly, cross-linking permitted determinations of aggregation responses under Ca2+-depleted conditions.

Sites of αβ3 aggregation were determined with both conventional and confocal microscopy. The addition of mAb LM609 alone resulted in diffuse fluorescence on the cell surface (Figures 1A and 1C). However, cross-linking of the mAb resulted in the formation of fluorescent clumps, signifying αβ3 aggregation in all viewed cells. The clumps were located largely at the cell periphery (Figure 1B) and were viewed best at the superficial confocal levels (Figure 1D). Similar clustering patterns occurred with the addition of vitronectin (400 μg/mL) and with cross-linking under external Ca2+-free conditions, indicating that the aggregation was not Ca2+-dependent.

[Ca2+]i Responses to Clustering αβ3

Figure 2 shows single cell images, exemplifying [Ca2+]i response patterns. Cross-linking (Figure 2, top) or the addition of multimeric vitronectin (not shown) caused [Ca2+]i increases that initiated at the cell periphery and then spread centripetally. In contrast, histamine-induced [Ca2+]i oscillations were usually evident in the recovery period. Cross-linking under control conditions caused a peak increase in [Ca2+]i, by 85±12 nmol/L (n=12;
In contrast, under Ca$^{2+}$-free conditions, cross-linking increased [Ca$^{2+}$]$_i$ by only 2765 nmol/L, indicating marked blunting of the response (n = 7; P < 0.01, n = 4).

Although cross-linking under Ca$^{2+}$-free conditions caused a blunted [Ca$^{2+}$]$_i$ increase, the subsequent addition of thapsigargin, the endosomal Ca$^{2+}$-ATPase inhibitor, markedly increased [Ca$^{2+}$]$_i$ (Figure 3D) (P < 0.05, n = 4). Because thapsigargin induces Ca$^{2+}$ release from endosomal stores, this result indicates that external Ca$^{2+}$-free conditions did not cause store depletion. However, when we first used thapsigargin to cause store depletion and then cross-linked monolayers in Ca$^{2+}$-free conditions, all [Ca$^{2+}$]$_i$ increases were blocked (Figure 3E; P < 0.05, n = 4). We interpret that clustering of the αβ3 integrin induced [Ca$^{2+}$]$_i$ transients arising from both the release of intracellular Ca$^{2+}$ stores and the influx of external calcium across the plasma membrane. Also shown are data from several experiments carried out to validate our procedures (n = 4 each). Thus, the intracellular Ca$^{2+}$-chelator MAPTAM (Figure 3F, 200 μmol/L) combined with mAb LM609–induced aggregation of αβ3. BPAEC monolayers fluorescently labeled with Cy3 conjugated with mAb LM609 were viewed after addition of buffer (A and C) or cross-linking IgG (B and D). Images by conventional (A and B) and confocal (C and D) microscopy of apical surfaces of single cells indicate aggregates (arrows). Replicated 3 times for each condition. LM609 indicates anti-αβ3 Ab; 2° Ab, secondary Ab (donkey anti-mouse IgG).
Protein Tyrosine Phosphorylation and [Ca\(^{2+}\)],
Regulation in BPAECs

In affirmation of our previous findings,\textsuperscript{17} cross-linking \(\alpha_\beta_3\), consistently enhanced tyrosine phosphorylation of several proteins, especially those corresponding to a kDa value of \(\approx 125, \approx 68, \approx 62, \approx 52, \approx 48,\) and \(\approx 34\) (Figure 5, lane 3). The addition of mAb LM609 alone had no effect (not shown). External Ca\(^{2+}\) removal, the addition of thapsigargin, and the addition of MAPTAM, the intracellular Ca\(^{2+}\) chelator, had no effects on the cross-linking–induced tyrosine phosphorylation of the 6 bands of interest (compare lanes 3, 5, and 7 in Figure 5). Hence, Ca\(^{2+}\) depletion did not affect tyrosine kinase activation relevant to these proteins.

When we combined external Ca\(^{2+}\) removal with either thapsigargin or MAPTAM, tyrosine phosphorylation decreased on the band at \(\approx 125\) kDa. External Ca\(^{2+}\) removal alone or in combination with thapsigargin or MAPTAM reduced tyrosine phosphorylation on several bands of less than 46 kDa (lanes 4, 6, and 8 in Figure 5). Hence, \(\alpha_\beta_3\)-induced tyrosine phosphorylation was Ca\(^{2+}\) independent for some, but not all, endothelial proteins.\textsuperscript{13}

We determined whether clustering the \(\alpha_\beta_3\) integrin on BPAECs induced enhanced tyrosine phosphorylation of PLC-\(\gamma_1\), which underlies InsP\(_3\)-induced Ca\(^{2+}\) fluxes evoked by other tyrosine kinase–mobilizing receptors.\textsuperscript{12} Immunoprecipitation experiments indicated that cross-linking \(\alpha_\beta_3\) enhanced tyrosine phosphorylation of PLC-\(\gamma_1\) and that the effect was blocked by the tyrosine kinase inhibitor genistein (100 \(\mu\)mol/L) (Figure 6A).\textsuperscript{29} We also determined that the addition of multimeric vitronectin or the clustering of cell surface \(\alpha_\beta_3\) led to modest but significant increases in InsP\(_3\) accumulation that were also abrogated by the addition of genistein (Figure 6B). Genistein also completely inhibited the [Ca\(^{2+}\)] increase in response to vitronectin (Figure 6C) as well as to cross-linking (not shown). Hence, the tyrosine kinase inhibitor blocked all 3 \(\alpha_\beta_3\)-induced effects: tyrosine phosphorylation of PLC-\(\gamma_1\), increased InsP\(_3\) production, and increased [Ca\(^{2+}\)].
To test for nonspecific inhibition of Ca\(^{2+}\) mobilization mechanisms, we determined that genistein did not inhibit histamine-induced [Ca\(^{2+}\)], increases, although it attenuated the effect. Thus, compared with control experiments in which histamine (10 \(\mu\)mol/L) increased [Ca\(^{2+}\)], by 80±12% above baseline (70±4 nmol/L) \((P<0.05, n=3)\), the response was attenuated in genistein-treated cells in which the increase was 30±7% \((P<0.05, n=3)\).

### Discussion

We reported previously that ligation of the lung endothelial \(\alpha_\beta_3\) integrin causes rapid enhancement of protein tyrosine phosphorylation.\(^{17}\) Here we show that an important consequence of this phosphorylation is the triggering of rapid [Ca\(^{2+}\)] increases. A comparison of our previous and present data indicates that the induced tyrosine phosphorylation and the [Ca\(^{2+}\)] increase, occurred with a similar time course. Importantly, PLC-1 was tyrosine phosphorylated and possibly activated as indicated by the increase in InsP\(_3\), a product of PLC-1-induced hydrolysis on inositol bisphosphate.\(^{12}\) The PLC-1 phosphorylation, the InsP\(_3\) increases, and the [Ca\(^{2+}\)], increases were completely inhibited with genistein. By contrast, histamine-induced [Ca\(^{2+}\)] increases occurred in the presence of genistein, indicating that genistein caused no nonspecific inhibition of Ca\(^{2+}\) mobilization. We conclude from these findings that interaction of the endothelial \(\alpha_\beta_3\) integrin with soluble ligands induced tyrosine phosphorylation as the primary mechanism for increasing endothelial [Ca\(^{2+}\)].

The [Ca\(^{2+}\)] increases were attributable in large part to Ca\(^{2+}\) entry, because they were 70% abrogated when external Ca\(^{2+}\) was depleted. However, a significant intracellular component was present, because the residual 30% of the response was blocked by prior treatment with thapsigargin, which depletes endosomal Ca\(^{2+}\) stores through inhibition of the endosomal Ca\(^{2+}\)-ATPase pump.\(^{24}\) Therefore, this intracellular component was likely due to Ca\(^{2+}\) release from thapsigargin-sensitive ER stores. InsP\(_3\) ligates endosomal receptors to cause store release of Ca\(^{2+}\). Further [Ca\(^{2+}\)] increases likely result from the Ca\(^{2+}\)-induced Ca\(^{2+}\)-release mechanisms,\(^{11}\) as well as by an inadequately understood mechanism via which store depletion causes entry of external Ca\(^{2+}\).\(^{30}\) As summarized in Figure 7, the present InsP\(_3\) increase is likely to have induced these [Ca\(^{2+}\)]-increasing mechanisms.

In the majority of cells, \(\alpha_\beta_3\)-induced [Ca\(^{2+}\)] increases first occurred at the cell margins and then spread centrally. This centripetal progression was not a phenotypic characteristic because histamine-induced [Ca\(^{2+}\)] increases occurred more uniformly. Although mechanisms are unclear, the peripheral initiation of the response may be in part due to the reported peripheral location of \(\alpha_\beta_3\).\(^{31,32}\) We confirmed \(\alpha_\beta_3\) aggregation through immunofluorescence (Figure 1). Light fixation of cells and permeabilization improved the detection of immunofluorescence but did not impair the ability of the integrin to undergo aggregation. Lawson et al\(^{23}\) also reported clustering of the \(\alpha_\beta_3\) integrin in lightly fixed cells. With the use of confocal microscopy, the immunofluorescent aggregates were best viewed on the luminal surface of the monolayer, indicating that the soluble ligands used probably aggregated luminal integrins. This may be expected because abluminal, matrix-facing integrins were probably already ligated to immobilized matrix elements.\(^{3}\) The aggregates were also located in large part at the cell periphery. Because cell contraction induced by [Ca\(^{2+}\)]-dependent processes opens interendothelial junctions,\(^{33}\) peripherally located \(\alpha_\beta_3\) integrins may promote endothelial monolayer permeability through the localization of [Ca\(^{2+}\)], increases to endothelial junctional regions.

[Ca\(^{2+}\)] responses to the \(\alpha_\beta_3\) integrin have been reported previously in freshly seeded, migrating endothelial cells.\(^{8}\) However, these reported responses differ from the present data in at least 2 important respects. First, in migrating cells, [Ca\(^{2+}\)], increases resulted entirely from the entry of external Ca\(^{2+}\), whereas here we determined a significant role for intracellular Ca\(^{2+}\) release. Second, migrating cells reached a peak response much more gradually, with delays of 20 to 30 minutes after plating, whereas here peak responses occurred in <2 minutes. These differences may be due to the fact that in freshly seeded cells, integrin interactions are probably determined by the extent and speed of cell spreading, which may prolong the [Ca\(^{2+}\)] response. The present short [Ca\(^{2+}\)], transients in stable endothelial cell monolayers may be more representative of vascular \(\alpha_\beta_3\) responses to circulating ligands.

Our findings address the role of protein tyrosine phosphorylation in receptor-mediated [Ca\(^{2+}\)] regulation. In addition to integrins and growth factor receptors, G protein–linked receptors also activate tyrosine kinases.\(^{13,14}\) In the presence of external Ca\(^{2+}\) depletion, the addition of MAPTAM or thapsigargin decreased tyrosine phosphorylation on several bands of <46 kDa. A similar endothelial effect was reported for bradykinin, which ligates a G protein–linked receptor.\(^{13}\) G protein–linked receptors are likely to first increase [Ca\(^{2+}\)], via the PLC-\(\beta\)/InsP\(_3\) mechanism, which is tyrosine kinase independent.\(^{12}\) However, this [Ca\(^{2+}\)], increase may activate Ca\(^{2+}\)-sensitive tyrosine kinases. The ensuing tyrosine phosphorylation may further increase [Ca\(^{2+}\)], via the PLC-\(\gamma\)/InsP\(_3\) mechanism.\(^{12}\) In this situation, genistein is expected to attenuate but not completely block the [Ca\(^{2+}\)], response, because it blocks only the tyrosine phosphorylation–dependent [Ca\(^{2+}\)], increase. Accordingly, we confirmed that unlike the complete inhibition of the [Ca\(^{2+}\)], increase to \(\alpha_\beta_3\),
cross-linking, genistein attenuated only the [Ca\textsuperscript{2+}] increase to histamine.\textsuperscript{34}

Our findings are also relevant to a consideration of the potential role of the endothelial αβ\textsubscript{3} integrin in general vascular responses. The present rapidly developed [Ca\textsuperscript{2+}], peak and subsequent [Ca\textsuperscript{2+}], oscillations are characteristic of inflammatory receptors. [Ca\textsuperscript{2+}], transients elicit processes leading to many types of cell function, such as secretion, endocytosis, and cell contraction.\textsuperscript{11,35,36} In endothelial cells, such transients have been associated with barrier deterioration,\textsuperscript{33} leukocyte adhesion,\textsuperscript{37} and cytokine secretion.\textsuperscript{6} In previous experiments, we reported increases in lung capillary permeability that are attributable to ligation of the αβ\textsubscript{3} integrin.\textsuperscript{4} D’Angelo et al\textsuperscript{10} recently reported that the integrin induces vasodilatation. The αβ\textsubscript{3} integrin recognizes apoptotic neutrophils\textsuperscript{38} and could contribute to endothelial margination of neutrophils.

These considerations indicate that although the biological significance of the endothelial αβ\textsubscript{3} integrin is usually discussed in relation to angiogenesis and vascular growth,\textsuperscript{2} the integrin may play a distinct role in vascular pathophysiological processes in general. Such a role may be particularly important in the lung, which appears to be an exception among vascular beds in that it expresses the αβ\textsubscript{3} integrin under resting, nonproliferative conditions.\textsuperscript{4,39} The αβ\textsubscript{3} integrin binds a wide range of both vitronectin-linked and nonlinked substances of potential pathophysiologic importance, such as SC5b-9, viruses, the thrombin-antithrombin complex, and malignant cells.\textsuperscript{17,21,22,40} This promiscuous binding property across the vast vascular surface of the lung, together with its ability to rapidly mobilize Ca\textsuperscript{2+}, accords the endothelial αβ\textsubscript{3} integrin a pathological potential that warrants further study.

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References

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