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Interplay between FAK, PKCδ, and p190RhoGAP in the Regulation of Endothelial Barrier Function

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Abstract

Disruption of either intercellular or extracellular junctions involved in maintaining endothelial barrier function can result in increased endothelial permeability. Increased endothelial permeability, in turn, allows for the unregulated movement of fluid and solutes out of the vasculature and into the surrounding connective tissue, contributing to a number of disease states, including stroke and pulmonary edema (Ermert et al., 1995; Lee and Slutsky, 2010; van Hinsbergh, 1997; Waller et al., 1996; Warboys et al., 2010). Thus, a better understanding of the molecular mechanisms by which endothelial cell junction integrity is controlled is necessary for development of therapies aimed at treating such conditions. In this review, we will discuss the functions of three signaling molecules known to be involved in regulation of endothelial permeability: focal adhesion kinase (FAK), protein kinase C delta (PKCδ), and p190RhoGAP (p190). We will discuss the independent functions of each protein, as well as the interplay that exists between them and the effects of such interactions on endothelial function.

Keywords

FAK; PKC; p190RhoGAP; endothelium; permeability; focal adhesions

Introduction

The endothelium forms the innermost lining of all body vessels. This single layer of cells functions as a semipermeable barrier, serving to regulate the exchange of fluid and solutes between the vascular compartment and the interstitial space (Deanfield et al., 2005). The integrity of this barrier is dependent upon the adhesions between adjacent endothelial cells, as well as the adhesions of the endothelial cells to the underlying extracellular matrix (Dejana et al., 1995; Dejana and Del Maschio, 1995). Interendothelial cell tight junctions and adherens junctions prevent uncontrolled paracellular transport of substances, including plasma proteins and white blood cells, through the endothelium and into the surrounding connective tissue. At the same time, numerous transmembrane protein complexes, known as focal adhesions, function in endothelial barrier regulation by modulating the attachment of individual endothelial cells to the underlying basement membrane. However, recent studies indicate that focal adhesions can also affect the signaling pathways that control cell survival

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and differentiation, as well as those involved in cell migration. In order to mediate such a diverse array of functions, signaling at focal adhesions occurs in a dynamic nature. Several molecules controlling protein phosphorylation and organization of the actin cytoskeleton are particularly crucial in focal adhesion function; included in these are focal adhesion kinase and protein kinase C, as well as the RhoA regulatory protein, p190RhoGAP.

Regulation of Endothelial Permeability by FAK, PKCδ, and p190RhoGAP

Focal Adhesion Kinase (FAK)—Focal adhesion kinase (FAK) is a ubiquitously expressed cytoplasmic protein tyrosine kinase involved in the regulation of numerous endothelial cell functions. It serves a critical role in vascular endothelial growth factor (VEGF)-induced angiogenesis and vascular patterning; mice with endothelial-specific deletion of FAK die prior to birth (Eliceiri et al., 2002; Shen et al., 2005). FAK is also involved in endothelial cell apoptosis, in the response to a number of barrier agonists (Bellas et al., 2002; Claesson-Welsh et al., 1998; Kabir et al., 2002), and in the regulation of endothelial permeability (Guo et al., 2005; Harrington et al., 2005; Holinstat et al., 2006).

As its name implies, FAK was first characterized according to its role at focal adhesion complexes (Schlaepfer and Hunter, 1996). Focal adhesions are comprised of combinations of between 50–100 different proteins, giving the structures considerable heterogeneity and dynamic signaling properties. However, all focal adhesions contain two major components: integrins and FAK (Sieg et al., 1999). Integrins are transmembrane glycoproteins which serve as tethers between the intracellular cytoskeleton and the protein components of the extracellular matrix. Each integrin molecule is composed of a heterodimeric pairing of various α and β subunits. To date, 18 α and 8 β subunits have been identified, giving rise to 24 possible integrin pairs, each with distinct adhesion receptor properties (Hynes, 2002). The integrin cytoplasmic domains mediate binding to actin filaments, either directly or indirectly through adaptor proteins including paxillin, vinculin, talin, and α-actinin. Integrin adhesion to underlying matrix components, such as vitronectin and fibronectin, is transduced through the integrin extracellular domains. It was traditionally thought that integrin-matrix engagement and subsequent integrin receptor clustering at sites of cell-extracellular matrix adhesion serves as the stimulus for recruitment of FAK (Mitra and Schlaepfer, 2006). However, recent data also suggests that FAK promotes the activation of integrins, resulting in altered force generation at cell-extracellular matrix interactions (Michael et al., 2009).

Upon integrin engagement, FAK is directed from the cytoplasm to sites of focal adhesions through a focal adhesion targeting (FAT) sequence, within its 140 amino acid carboxyl-terminus (Hildebrand et al., 1993). The amino-terminal FERM (protein 4.1, ezrin, radizin, and moesin homology) domain functions to maintain FAK in an autoinhibited state by masking the catalytic domain (Lietha et al., 2007), as well as to facilitate the physical binding of FAK to integrin proteins, certain growth factor receptors, and the actin polymerizing protein complex, Arp2/3 (Chen and Chen, 2006; Serrels et al., 2007; Sieg et al., 2000). Integrin binding induces the autophosphorylation of FAK at tyrosine 397 (Hamadi et al., 2005; Hanks and Polte, 1997), within the carboxyl-terminal region, which in turn facilitates binding to the SH2 domains of Src or phosphatidylinositol 3-kinase (PI 3-kinase). The FAK protein complex then mediates the phosphorylation and activation of several key molecules important in cell adhesion and migration, including paxillin and $p130^{Cas}$ (Mitra and Schlaepfer, 2006; Schaller et al., 1994; Vuori, 1998). When FAK is molecularly inhibited or ablated, cell spreading and stress fiber formation is inhibited and nuclei become condensed and lobular (Almeida et al., 2000; Ilic et al., 1998; Ilic et al., 1995; Richardson et al., 1997; Sieg et al., 1999).

In addition to its role in cell-extracellular matrix adhesion, FAK has more recently been shown to contribute to endothelial cell-cell adhesion. The vascular barrier agonist,

sphingosine 1-phosphate (S1P), a sphingolipid which has been shown to enhance vascular endothelial integrity (Garcia et al., 2001), induces a redistribution of FAK, and its binding partner paxillin, from internal sites of extracellular matrix adhesion to the cell periphery (Sun et al., 2009). Once at the cell periphery, FAK associates with VE-cadherin-mediated adherens junctions through its binding to β-catenin and p120-catenin (Knezevic et al., 2009). This interaction between FAK and adherens junction proteins fosters the establishment of the cortical actin ring. FAK was also shown to play a critical role in the reannealing of adherens junctions following settings of endothelial barrier dysfunction. PAR1 receptor activation via thrombin was shown to promote the release of the heterotrimeric $G_{\beta\gamma}$ subunit from its sequestering protein, receptor for activated C kinase 1 (RACK1), and subsequent binding to and activation of Fyn and FAK (Knezevic et al., 2009). This sequence of events resulted in FAK binding to p120-catenin and reannealing of adherens junctions (Knezevic et al., 2009). By transiently overexpressing various forms of FAK, others have also demonstrated cross-talk between FAK and adherens junctions in the regulation of endothelial barrier function (Quadri and Bhattacharya, 2007; Usatyuk and Natarajan, 2005). In a recent study in which a kinase defective FAK protein that was conditionally knocked into the endothelium, it was shown that, while kinase dead FAK is protective against endothelial cell apoptosis, endothelial cell barrier function and the intercellular localization of VE-cadherin at the adherens junctions is dependent upon FAK kinase activity (Zhao et al., 2010). In addition, activation of the endothelial cell-specific, extracellular matrix bound integrin, $\alpha_v\beta_3$, caused the redistribution of this integrin to the cell periphery, diminution of VE-cadherin at inter-endothelial cell junctions, FAK activation, and concomitant barrier dysfunction (Alghisi et al., 2009). Thus FAK is a key regulator of endothelial barrier function at both integrin-mediated cell-extracellular matrix interactions, as well as intercellular adherens junctions.

Protein kinase C—Protein kinase C (PKC) is a family of serine/ threonine kinases consisting of ten known isoforms. These isoforms are divided into three subfamilies, based on their domain composition and corresponding cofactor requirements (Newton, 1995). All of the PKC enzymes possess a highly conserved carboxyl-terminal kinase domain which is autoinhibited by the binding of the amino-terminal pseudosubstrate domain within the substrate cleft of the catalytic domain. The PKC isoforms are catalytically activated upon: cofactor binding; sequential phosphorylation of select serine or threonine residues by phosphoinositide-dependent kinase-1 (PDK-1) and mammalian target of rapamycin (mTOR) complex 2 (mTORC2); and/ or allosteric interactions with key effector proteins (Newton, 2010; Rosse et al., 2010). The first subfamily, the conventional (c) PKC enzymes, is comprised of the α, β_I, β_{II}, and γ isoforms. Within the N-terminal regulatory region, these enzymes contain a C1 domain, which binds either diacylglycerol (DAG) or the pharmacologic analogue, phorbol ester. The C2 domain binds to the head groups of phospholipids, such as phosphatidylserine, in the presence of calcium. In order to be activated, both the C1 and C2 domains must be engaged, thus requiring the presence of calcium, diacylglycerol (DAG), and a phospholipid. The second group of PKC enzymes, the novel (n) family, is comprised of the δ, ε, η, and θ isoforms. Like the cPKC isoforms, activation of the nPKC enzymes requires binding to DAG/ phorbol esters, as well as to a phospholipid; however, due to a variation in the amino acid sequence of the C2 domain, the presence of calcium is not necessary. The third subfamily of PKC enzymes contains the atypical (a) isoforms, ζ and ι/λ. The aPKC enzymes, which lack functional C1 and C2 domains, do not require DAG or calcium for activation. Instead, aPKC are activated primarily by protein-protein interactions with the partitioning defective 6 (PAR6)-CDC42 protein complex (Suzuki et al., 2001). Much work has been done to elucidate signaling pathways upstream and downstream of the PKC isoforms since their discovery in the 1980s, however substrate specificity of each of the isoforms is still not known.

Inhibitor studies have suggested proteins associated with the cytoskeleton or adherens junction may serve as substrates for PKC and thus may be important in the modulation of thrombin-induced changes in endothelial monolayer permeability. Studies examining signaling molecules important in inducing lung edema showed that infusion of PKC activating agents induced edema formation, while infusion of PKC inhibitors blocked the effects of edemagenic agents in lung edema formation (Johnson et al., 1990; Johnson et al., 1989; Siflinger-Birnboim et al., 1992). More recently, phorbol ester-induced microvascular endothelial monolayer permeability was shown to require PKCδ, but not PKCα, β_I, or ε (Tinsley et al., 2004). Others have demonstrated increased endothelial barrier dysfunction upon modulation of PKCα, PKCβ_I, or PKCζ activities and/or expression (Ferro et al., 2000; Huang et al., 2005; Li et al., 2004; Nagpala et al., 1996). PKCα activation promoted endothelial barrier disruption in response to a variety of edemagenic agents, including α-thrombin, TNF-α, and ROS (Aschner et al., 1997; Ferro et al., 2000; Konstantoulaki et al., 2003; Sandoval et al., 2001; Xiong et al., 2010). Intriguingly, we have shown that activation of PKCδ is critical for maintenance of basal barrier function and attenuated agonist-induced increases in permeability (Figure 1) (Harrington et al., 2003; Harrington et al., 2005; Klinger et al., 2007); these functions correlated with enhanced focal adhesion formation, actin filament stabilization, and RhoA activation. Similarly, using a siRNA approach, Carpenter and Alexander demonstrated that PKCδ activation within endothelial cells attenuated neutrophil transmigration across the monolayers (Carpenter and Alexander, 2008). Additional studies have suggested that diminished PKCδ expression and upregulation of PKCβ_{II} protein content in the endothelium may contribute to microvascular barrier dysfunction in settings of hyperglycemia, possibly through distinct subcellular compartmentalization of each isoform (Gaudreault et al., 2008; Yuan et al., 2000). Indeed, a recent study using a PKCδ selective fluorescence resonance energy transfer (FRET) reporter construct which reflected PKCδ enzymatic activity, showed that PKCδ activity was greatest at the plasma membrane in COS7 cells at both baseline and in agonist-induced states; data supportive of a role of PKCδ in modulating barrier function (Kajimoto et al., 2010). Interestingly, in a recent study, Geraldes and colleagues demonstrated an indirect effect of PKCδ in endothelial cell dysfunction via the induction of apoptosis. The investigators noted that hyperglycemia induced the upregulation of PKCδ dependent signaling in pericytes, which in turn led to pericyte apoptosis and microvascular dysfunction via increased endothelial permeability and cell proliferation, and the pathologic progression of diabetic retinopathy (Geraldes et al., 2009).

Many agents that affect endothelial barrier function similarly modulate proliferation, migration, and angiogenesis. Thus it is not surprising that PKC isoforms are critical regulators of these endothelial functions (Anfuso et al., 2007; Graham et al., 2000; Hu and Fan, 1995; Spyridopoulos et al., 2002; Wong and Jin, 2005). PKCδ overexpression reduced endothelial cell proliferation via a diminished rate of progression through the G1/S-phase of the cell cycle and an attenuated level of expression of cyclin E (Ashton et al., 1999; Harrington et al., 1997). Further experiments have demonstrated that conditional overexpression of the PKCη pseudosubstrate motif, which inhibited PKCα, δ, ε, and η enzymatic activities *in vitro*, also attenuated the rate of endothelial cell proliferation, migration, and tubule formation (Harrington et al., 2000). We showed PKCδ overexpression enhanced endothelial cell adhesion to the extracellular matrix protein, vitronectin (Harrington et al., 1997). However, isoforms other than PKCδ have been shown to be necessary for the regulation of endothelial cell migration induced by various agents, including sphingosine-1-phosphate, hepatocyte growth factor, vascular endothelial growth factor (VEGF) (Gorshkova et al., 2008; Harrington et al., 1997; Wang et al., 2002).

Multiple studies have revealed a pro-apoptotic role for PKCδ in the progression of apoptosis *in vitro* and *in vivo*. PKCδ activation was shown to be associated with increased apoptosis in

myocardial tissue of patients undergoing cardiopulmonary bypass and carioplegic arrest (Sodha et al., 2008). Furthermore, the PKCδ-specific translocation peptide inhibitor, KAI-9803, was shown to attenuate the infarct size in animal models and in humans with acute myocardial infarction (Direct Inhibition of δ-Protein Kinase C Enzyme to Limit Total Infarct Size in Acute Myocardial Infarction (DELTA MI) Investigators, 2008; Inagaki et al., 2003). Furthermore, the salivary glands of PKCδ null mice were resistant to ionizing radiation-induced apoptosis (Humphries et al., 2006). Interestingly, PKCδ has also been shown to protect against apoptosis (Kilpatrick et al., 2006). In epithelial cells, PKCδ promoted cell survival in response to acute hypoxia via activation of the autophagic response (Humphries et al., 2006). However, upon chronic hypoxic conditions, apoptosis ensues via a mechanism involving the catalytically-active form of PKCδ (PKCδCF) and caspase-3 (Humphries et al., 2006). Studies by Reyland and colleagues have demonstrated that upon exposure to apoptosis-inducing agents, PKCδ phosphorylation at tyrosine residues 64 and 115 causes a protein conformational change revealing a nuclear localization sequence, which in turn resulted in the translocation of PKCδ to the nucleus (DeVries-Seimon et al., 2002; DeVries-Seimon et al., 2007). Thus, it is likely that different subcellular compartments of PKCδ regulate distinct cellular functions; including monolayer permeability, proliferation, apoptosis, migration, adhesion, and angiogenesis. The maintenance of the vascular integrity requires a careful balance of signals regulating endothelial cell survival and apoptosis, thus it is possible that endothelial cell apoptosis plays a role in edema formation seen in settings of chronic obstructive pulmonary diseases and acute lung injury.

The expression pattern of the PKC isoforms varies in endothelial cells depending upon the organ or vascular bed from which the cells are isolated (Geraldes et al., 2009). For example, in the lung, we noted the PKCα, δ, ε, η, and λ isoforms in microvascular endothelial cells and only PKCα, δ, ε, and η in pulmonary artery derived endothelial cells (Fordjour and Harrington, 2009). Because PKC has been implicated in a variety of endothelial cell functions, including monolayer permeability, proliferation, apoptosis, migration, adhesion, and angiogenesis, it is likely that the PKC isoform profile of the endothelium may also influence the functional and pathologic response of the endothelial cell to environmental cues.

p190RhoGAP—The monomeric, small Rho GTPase proteins (RhoA, Rac-1, and Cdc-42) are members of the Ras superfamily and are key cytoskeletal regulators, functioning in cell motility and migration, cytokinesis, differentiation, polarity, and vesicular trafficking (Aspenstrom, 1999; Begum et al., 2004; Hall, 1998; Hall and Nobes, 2000; Hotchin and Hall, 1996; Ridley, 2001; Tapon and Hall, 1997; Wojciak-Stothard and Ridley, 2002a). These ubiquitous enzymes cycle between active, GTP-bound states and inactive, GDP-bound states, undergoing corresponding conformational changes. The exchange of GTP and GDP is regulated by the opposing actions of the guanine nucleotide exchange factors (GEF) and the GTPase activating proteins (GAP). The GEF proteins mediate the activation of RhoA, Rac-1, and Cdc-42 by stimulating the release and exchange of GDP for GTP. Conversely, the GAP proteins foster the inactivation of these proteins by activating their intrinsic GTPase activity, triggering the conversion from the active, GTP-bound state to the inactive, GDP-bound form. Another class of proteins, the Rho guanine nucleotide dissociation inhibitors (GDI) suppress RhoA signaling by sequestering the GDP-bound form of the small GTPases within the cytoplasm, preventing them from exposure to and activation by the GEF proteins (Fukumoto et al., 1990; Moon and Zheng, 2003).

The p190RhoGAP (p190) family of GTPase activating proteins is composed of two members: p190RhoGAP-A (p190-A) (Settleman et al., 1992) and p190RhoGAP-B (p190-B) (Burbelo et al., 1998). While both proteins function as GAP for the Rho family of small

GTPases, they are encoded by different genes and share only about 50% sequence identity. The two isoforms do exhibit several overlapping functions, including the regulation of cortical actin assembly/disassembly and cell motility and invasion. p190-B, however, functions largely in regulation of development (Chakravarty et al., 2003; Heckman et al., 2007; Sordella et al., 2002) and cell-fate decisions (Sordella et al., 2003), while p190A is a critical regulator of cell migration and tumorigenesis in numerous cell types, including endothelial cells (Arthur et al., 2000; Wolf et al., 2003).

p190-A is comprised of three major domains: an N-terminal GTP-binding domain; a middle domain containing multiple protein-protein interaction sites, including several diphenylalanine (FF) motifs shown to interact with RNA-binding proteins (Jiang et al., 2005; Mammoto et al., 2009), numerous Src homology 3 (SH3)-domain binding sites, and a critical tyrosine residue, Tyr 1105, which, when phosphorylated, serves as the major binding site for p120RasGAP (Hu and Settleman, 1997; Roof et al., 1998); and a C-terminal GAP domain, which displays specificity for GTP-bound RhoA (Ridley et al., 1993).

p190-A plays a critical role in extracellular matrix dependent RhoA inhibition. Fibroblasts transiently overexpressing dominant negative p190 are unable to suppress RhoA during adhesion, leading to impaired cell spreading and migration (Arthur and Burridge, 2001). p190-A is also involved in regulation of adherens junction integrity, through its binding to p120-catenin. Activated RhoA disrupts adherens junction integrity by inducing myosin light chain-dependent actin stress fiber formation and initiating cytoskeleton retraction. While both p190-A and p120-catenin function independently to inhibit RhoA (p120-catenin functions as a modified GDI), the two also function in concert with one another to inhibit RhoA through Rac-1. Overexpression of dominant active Rac-1 induces a robust recruitment of p190 to adherens junctions, an effect which is blocked by depletion of p120-catenin. Likewise, p190-null cells lack adherens junction-associated p120-catenin, even in the presence of dominant active Rac-1 (Wildenberg et al., 2006).

In the endothelium, some evidence has suggested p190 is a key signaling modulator of endothelial barrier function. Angiopoietin-1 attenuation of lipopolysaccharide-induced endothelial barrier dysfunction *in vitro* and lung edema *in vivo* was shown to be blocked in settings of p190 protein suppression, suggesting that p190 signals through a barrier protective mechanism (Mammoto et al., 2007). In lung endothelial cells transiently overexpressing wild-type p190-A, while the endothelial cells remained adherent, we noted diminished actin stress fibers and significantly fewer focal adhesion complexes (Fordjour and Harrington, 2009). Interestingly, transient overexpression of dominant negative p190-A protein had no significant effect on endothelial stress fiber or focal adhesion complex formation and endothelial basal barrier function was unaffected upon siRNA suppression of p190-A and/or p190-B (Fordjour and Harrington, 2009), suggesting that other Rho GAP proteins may compensate for these cytoskeletal disruptions in p190 function within the endothelium. Thus, p190 may play a greater role in the regulation of endothelial monolayer permeability in the settings of barrier agonists and antagonists.

Functional Crosstalk between FAK, PKCδ, and p190

Despite the considerable phenotypic and functional heterogeneity between macrovascular and microvascular endothelial cells, numerous *in vitro* studies suggest that FAK, PKCδ, and p190-A play similar roles in regulation of barrier function in both cell types (Mehta, 2002; Harrington, 2005; Holinstat, 2006). However, the baseline permeability of macrovascular endothelium, such as that derived from the pulmonary artery, is considerably higher than that of microvascular endothelium, with macrovessel-derived endothelial monolayers exhibiting increased hydraulic conductance, relative to endothelial cells derived from microvessels (Parker, 2006). Several reasons have been implicated for this difference,

including a differential response to intracellular Ca^{2+} flux and varying extracellular milieu (Kelly, 1998; Sisbarro, 2005). Interestingly, when pulmonary artery endothelial cells and lung microvascular endothelial cells were plated onto uncoated plastic, in order to mimic the microenvironment of injured tissue, macrovessel-derived cells displayed increased FAK activation and decreased RhoA activity, compared to microvascular endothelial cells (Sisbarro, 2005). Thus, it is likely that while the cellular function of these molecules is similar in macrovascular and microvascular endothelium, their relative levels of expression may differ depending on the specific microenvironment.

PKCδ and FAK are involved in the regulation of several common endothelial cell functions; thus it is perhaps not surprising that there exists a certain degree of crosstalk between the two. PKCδ, in addition to several other PKC isoforms, is activated upon integrin ligation, leading to translocation from the cytosol to the cell membrane (Besson et al., 2002; Chae et al., 2010). Inhibition of the PKC enzymes has been shown to prevent cell spreading and migration (Chae et al., 2010; Wang et al., 2002). In rat embryonic fibroblasts, PKCδ has been shown to be one of the first components recruited to newly formed focal adhesions, rapidly following recruitment of FAK (Barry and Critchley, 1994). Overexpression of PKCδ has been shown to enhance endothelial barrier integrity and to attenuate the degree to which thrombin induces barrier dysfunction (Harrington et al., 2003). These observations were accompanied by a significant increase in the number of FAK-based focal adhesion contacts (Harrington et al., 2003). Conversely, inhibition of PKCδ significantly reduced the number and size of focal adhesions and diminished level of filamentous actin; events which correlated with attenuation of FAK activity and diminished cell stiffness, respectively (Figure 2) (Harrington et al., 2005; Klinger et al., 2007). Interestingly, attenuation of FAK activity was not detectable until ten minutes after treatment with the PKCδ inhibitor, rottlerin, and overexpression of wild type FAK was unable to block rottlerin-induced effects on endothelial permeability and stress fiber disruption, strongly suggesting that the effects of PKCδ on FAK are mediated through at least one intermediate signaling molecule, and/ or that PKCδ itself serves as an signaling intermediate for activation of FAK. Autophosphorylation of FAK at tyrosine 397 has been shown to occur downstream of RhoA activation (Mukai et al., 2003); thus, it is possible that PKCδ activates RhoA, which in turn stimulates autophosphorylation and activation of FAK (Figure 4). Alternatively, under homeostatic conditions, PKCδ may serve as signaling intermediate for one of the established modulators of FAK autophosphorylation, such as EGF or c-Met (Kharait et al., 2006; Thors et al., 2003; Wang et al., 2009).

Neutrophil transendothelial migration has been shown to induce changes in the phosphorylation of endothelial FAK, leading to an increase in vascular permeability (Guo et al., 2005). Treatment of endothelial cells with either phorbol 12-myristate 13-acetate (PMA), a known pan PKC activator, or bryostatin-1, an activator specific for PKCδ and PKCε, were shown to significantly attenuate neutrophil transendothelial migration, coincident with increased cell substrate adhesion and increased intracellular staining for the activated, phosphorylated form of FAK, phosphorylated tyrosine 397 (Y^{397}) FAK (Carpenter and Alexander, 2008). Although the mechanism by which PKCδ may regulate FAK has not yet been elucidated, PKCδ activity has been shown to increase following phosphorylation by Fyn kinase, a known activator of FAK (Kronfeld et al., 2000). Chu et al. recently reported an increase in FAK serine 910 (S^{910}) phosphorylation in rat ventricular myocytes transfected with constituitively active PKCδ (Chu et al., 2010). They also observed an inhibition of endothelin-1-induced FAK S^{910} phosphorylation in cells overexpressing dominant negative PKCδ. Endothelin is an important vasoactive mediator associated with numerous cell responses, including the release of nitric oxide from vascular endothelial cells. Thus, further investigation into the mechanism(s) by which PKCδ affects changes in FAK activation/

phosphorylation will serve to increase our understanding of the pathways involved in endothelial barrier regulation.

Coincident with their critical role in cytoskeletal organization and actin dynamics, the small Rho GTPase proteins are tightly regulated. Basal activation of RhoA is necessary for homeostatic endothelial function (Etienne-Manneville and Hall, 2002); increased RhoA activation, however, has been shown to correlate with endothelial cell migration and metastasis (Della Peruta et al., 2010; van Nieuw Amerongen et al., 2003; Zhao et al., 2006), increased neutrophil extravasation (Adamson et al., 1999; Wittchen et al., 2005; Worthylake and Burridge, 2003; Worthylake et al., 2001), and is the key mediator of endothelial cell contraction, coincident with increased vascular permeability (Carbajal and Schaeffer, 1999; McKenzie and Ridley, 2007; Partridge et al., 1992; Wojciak-Stothard and Ridley, 2002b). Thrombin, an established edemagenic agent, causes a very rapid and robust increase in endothelial RhoA activation, correlating with decreased endothelial barrier function (van Nieuw Amerongen et al., 2000).

Interestingly, thrombin also induces the recruitment of FAK to focal adhesions, where it has been implicated to function in the recovery of the endothelial barrier following disruption; endothelial monolayers in which FAK has been depleted are unable to restore barrier function following thrombin treatment (Mehta et al., 2002). Based on several independent observations that: 1) deletion of FAK in fibroblasts led to an increase in RhoA activation; 2) increased RhoA signaling correlated with increased endothelial permeability; 3) p190 co-localized with FAK at focal adhesions; and 4) p190 is activated by tyrosine phosphorylation, Hollinstat and colleagues (Holinstat et al., 2006) further investigated the specific role of FAK in regulation of endothelial RhoA activation. They noted increased tyrosine phosphorylation of p190 in response to thrombin, correlating with decreased RhoA activation. In addition, they demonstrated FAK phosphorylation of p190 *in vitro*, and showed that inhibition of FAK resulted in decreased p190 activation and a concomitant increase in RhoA activity and endothelial permeability. More recently, FAK/ p190 signaling was also implicated in mediating the anti-proliferative/ antiangiogenic effects of the combustion byproduct 3-methylcholanthrene (3MC), an aryl-hydrocarbon receptor agonist, in human umbilical vein endothelial cells (HUVEC) (Pang et al., 2008). Treatment with this compound resulted in a down-regulation of FAK, coincident with increased RhoA activation; this was in turn correlated with suppression of p190 activation by FAK (Chang et al., 2009). In addition, Tomar and colleagues demonstrated that a complex of FAK, p120RasGAP, and p190 functions to regulate the polarity and cell migration of fibroblasts, carcinoma cells, and endothelial cells (Tomar et al., 2009). In this study, they observed that following fibronectin-integrin engagement, activated FAK binds to p120RasGAP, through its SH2-SH3-SH2 region. Through this same region, p120RasGAP binds to p190-A, facilitating its activation by FAK. The FAK-p120RasGAP-p190A complex is then targeted to leading-edge focal adhesions, permitting spatially regulated RhoA suppression at cell protrusions. The authors also observed that overexpression of p190 mutants either lacking GAP activity (p190A-RA) or with key tyrosine residues mutated to phenylalanine (p190A-FF; Y1087F, Y1105F) blocked cell polarization, supporting the involvement of these p190-A domains in its interaction with FAK and p120RasGAP. Interestingly, PKCδ, which immunoprecipitates with p120RasGAP (Harrington et al., 2005), has been shown *in vitro* to bind to these two domains of p190-A (Fordjour and Harrington, 2009), suggesting a possible interplay of FAK, p190-A, and PKCδ.

p190-A also serves as a requisite binding partner for p120-catenin at intercellular adherens junctions, preventing its translocation to the cytoplasm (Wildenberg et al., 2006). Given the reported interaction between FAK and adherens junction associated p120-catenin (Sun et al.,

2009), it appears that p190-A is essential for FAK function not only at focal adhesions but also at adherens junctions.

While both PKCδ and p190-A function in regulation of endothelial FAK signaling, it appears that the two may serve differential functions in settings of barrier maintenance versus settings of barrier recovery after insult. For example, it was previously reported that, in addition to causing diminished FAK activation, inhibition of PKCδ also caused a concomitant decrease in baseline RhoA activity, resulting in disruption of basal endothelial barrier function (Harrington et al., 2005). Furthermore, PKCδ has been shown to co-immunoprecipitate with both p120RasGAP and p190 (Harrington et al., 2005). However, while PKCδ is able to regulate p190 activity (Figure 3), suppression of p190-A or p190-B, either independently or in combination, was unable to attenuate the effects of PKCδ inhibition on RhoA activity, focal adhesion disruption, stress fiber formation, or endothelial permeability in unstimulated cells (Fordjour and Harrington, 2009); this suggests that, under baseline conditions, PKCδ functions to regulate RhoA activity independently of p190. However, Holinstat and colleagues demonstrated that FAK signaling through p190 and subsequent inhibition of RhoA was critical for restoration of the pulmonary endothelium after thrombin-induced injury (Holinstat, 2006). Thus, as outlined in Figure 4, it is likely that the activities of both PKCδ and p190 differ depending upon the state of the endothelium. PKCδ may function to maintain basal levels of RhoA activation independently of p190 when the endothelial barrier is intact. In this setting, PKCδ may phosphorylate p190, facilitating its association with FAK, p120RasGAP, and potentially p120-catenin, without affecting its ability to inhibit RhoA activity, which presumably should remain at homeostatic levels. However, when the barrier is disrupted, as occurs following exposure to thrombin, and RhoA activation is elevated to supraphysiologic levels, PKCδ signaling may be acutely suppressed, providing a feedback loop preventing further activation of RhoA and potentially alleviating serine/ threonine phosphorylation of p190.

Given the critical role of FAK in regulating endothelial cell function, a greater understanding of the cellular proteins involved in FAK activation and downstream signaling, including PKCδ and p190, will help to gain insight into the mechanisms responsible for endothelial dysfunction in various disease states (Figure 4). Further investigation into the dynamic interactions of adherens junction-associated p120-catenin with FAK and its binding partner p120RasGAP may reveal a potential signaling axis along which signaling molecules, such as PKCδ and p190, serve to maintain the balance between endothelial cell-ECM adhesions and intercellular interactions necessary for vascular barrier function. Once the functions of these molecules with regard to FAK have been elucidated, as well as the temporal nature in which they are altered in different physiological settings, targeted therapies may be designed with the goal of attenuating endothelial injury and result barrier disruption.

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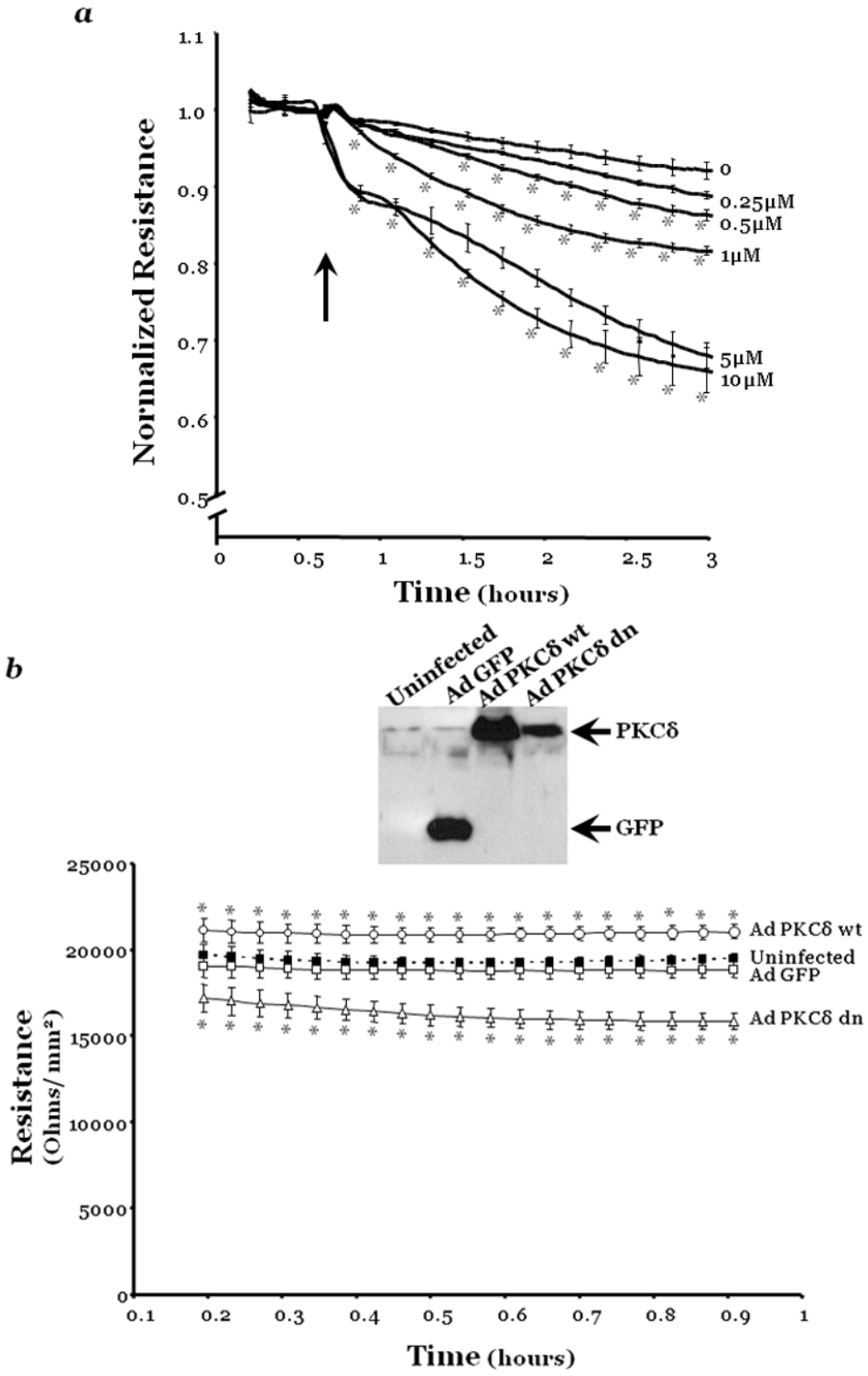
RESEARCH HIGHLIGHTS

- FAK, PKC, and p190RhoGAP exhibit crosstalk in their regulation of endothelial cell (EC) function.
- Changes in PKCδ correlate with alterations in FAK activity and related effects on EC function.
- FAK inhibition decreases p190RhoGAP activation; whereas PKCδ inihibition increases p190RhoGAP activation.
- PKCδ and p190RhoGAP may regulate FAK differentially in settings of barrier maintenance versus recovery.



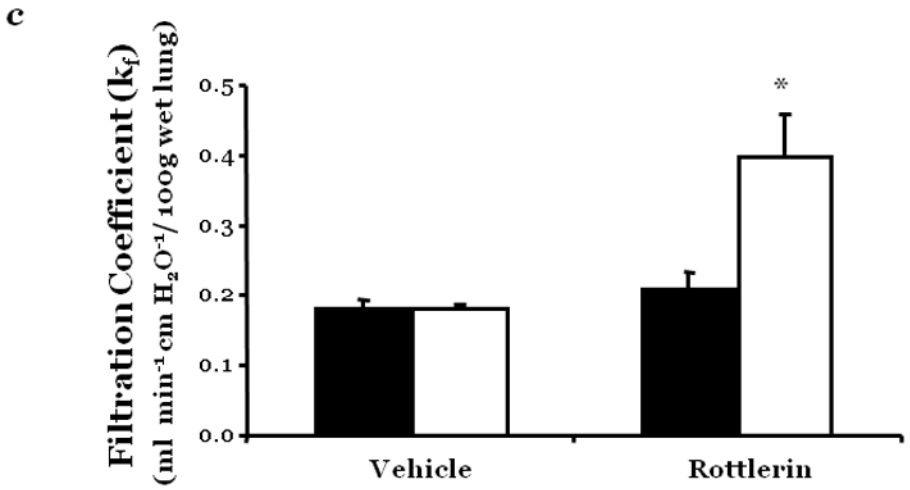


Figure 1. PKCδ inhibition promotes endothelial barrier dysfunction

Changes in endothelial monolayer permeability were assessed in rat lung microvascular endothelial cells (LMVEC) (*panel a*) or endothelial cells derived from the epididymal fat pad (FPEC) (*panel b*) by assaying changes in resistance of endothelial monolayers grown on collagen coated gold electrodes using the electrical cell impedance system (ECIS); a drop in electrical resistance across the endothelial monolayers correlates with increased permeability. *Panel a*, vehicle (DMSO) or indicated concentration of rottlerin (a chemical inhibitor with specificity for PKCδ, relative to other PKC isoforms) was added to the monolayers, with arrows indicating time of addition. *Panel b*, endothelial monolayers containing equivalent numbers of endothelial cells were infected with indicated adenovirus. Protein overexpression was confirmed by immunoblot analyses (inset) and the effect of the overexpressed protein on monolayer permeability was determined by measuring the electrical resistance across the monolayers 24 hours post-infection. The mean±SE of the normalized electrical resistance are presented. *Panels a*, n=6–12; *p<0.05 vs. vehicle. *Panel b*, n=16; *p<0.05 vs. Ad GFP or uninfected. *Panel c*, pulmonary vascular permeability was measured by calculating the capillary filtration coefficients (K_f), using the Starling equation, from isolated, perfused rat lungs, which were fully recruited and in an isogravametric state. K_f was determined by measuring the lung weight gain following an increase in venous pressure divided by the change in capillary pressures and normalized to 100g wet lung mass at baseline (solid bars) and following a 45 minute exposure to vehicle (DMSO) or 50μM rottlerin (open bars). n=3–4, *p<0.05.

Panels a, c, and *d*: Reprinted from Klinger, J.R., et al., 2007. Rottlerin causes pulmonary edema in vivo: A possible role for PKCδ. *Journal of Applied Physiology*, 103:2084–2094.
Panel b: Reprinted from Harrington, E.O., et al., 2005. PKCδ regulates endothelial basal barrier function through modulation of RhoA GTPase activity. *Experimental Cell Research*, 308:407–421.

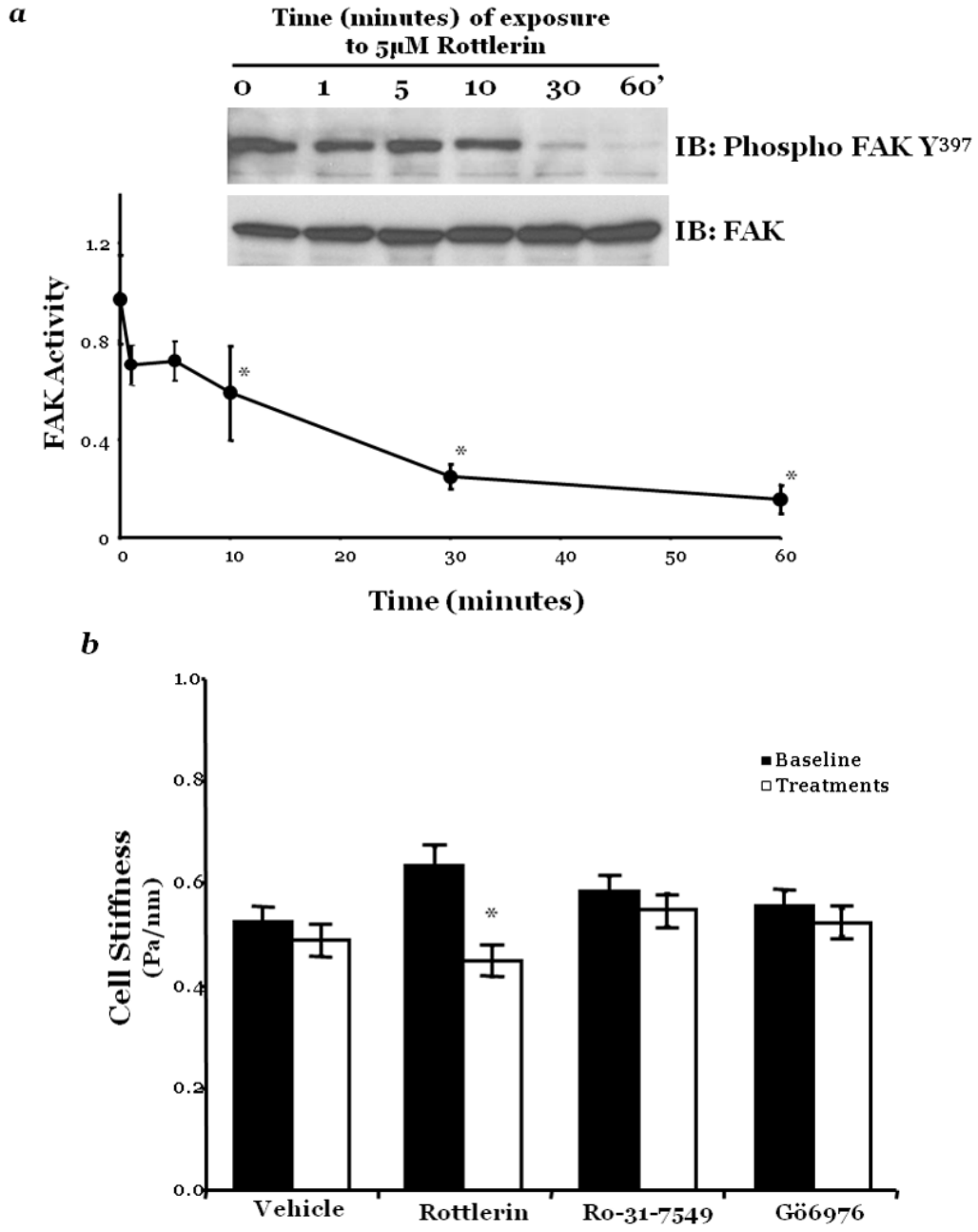


Figure 2. PKCδ inhibition blunts FAK activity and diminished cytoskeletal stiffness

Panel a, FAK activity was determined by measuring the level of phosphorylation at FAK Y^{397} by immunoblot analysis at indicated times following incubation of endothelial cells derived from the epididymal fat pad (FPEC) with 5μM rottlerin. The immunoblotted membranes were subsequently stripped and reprobed for FAK. Immunoblot signals were quantitated by densitometry and the level of FAK activity is presented as the mean±SE of the ratio of FAK Y^{397} phosphorylation to total FAK. *Panel b*, barrier function is dictated by changes in both contractile and adhesive forces, thus to measure changes in the contractile forces, cytoskeletal stiffness was assessed in lung microvascular endothelial cells (LMVEC) which were overlaid with ferrimagnetic beads, coated with the integrin receptor-specific peptide sequence (Arg-Gly-Asp; RGD), forming apical focal adhesions between the

LMVEC and the ferrimagnetic beads. The beads were then twisted, using a magnet, and the resistant force was measure both before treatment (i.e., baseline) and in the same cultures 30 minutes following exposure to vehicle, 250nM Ro-31-7549 (a chemical inhibitor with specificity for PKCα, β, γ, ε), 10nM Gö6976 (a chemical inhibitor with specificity for PKCα, β, γ), or 5μM rottlerin. Data are presented as mean±SE (n=280–520 cells). *$p<0.05$ vs. vehicle with respective treatment. *Panel a*: Reprinted from Harrington, E.O., et al., 2005. PKCδ regulates endothelial basal barrier function through modulation of RhoA GTPase activity. *Experimental Cell Research*, 308:407–421. *Panel b*: Reprinted from Klinger, J.R., et al., 2007. Rottlerin causes pulmonary edema in vivo: A possible role for PKCδ. *Journal of Applied Physiology*, 103:2084–2094.

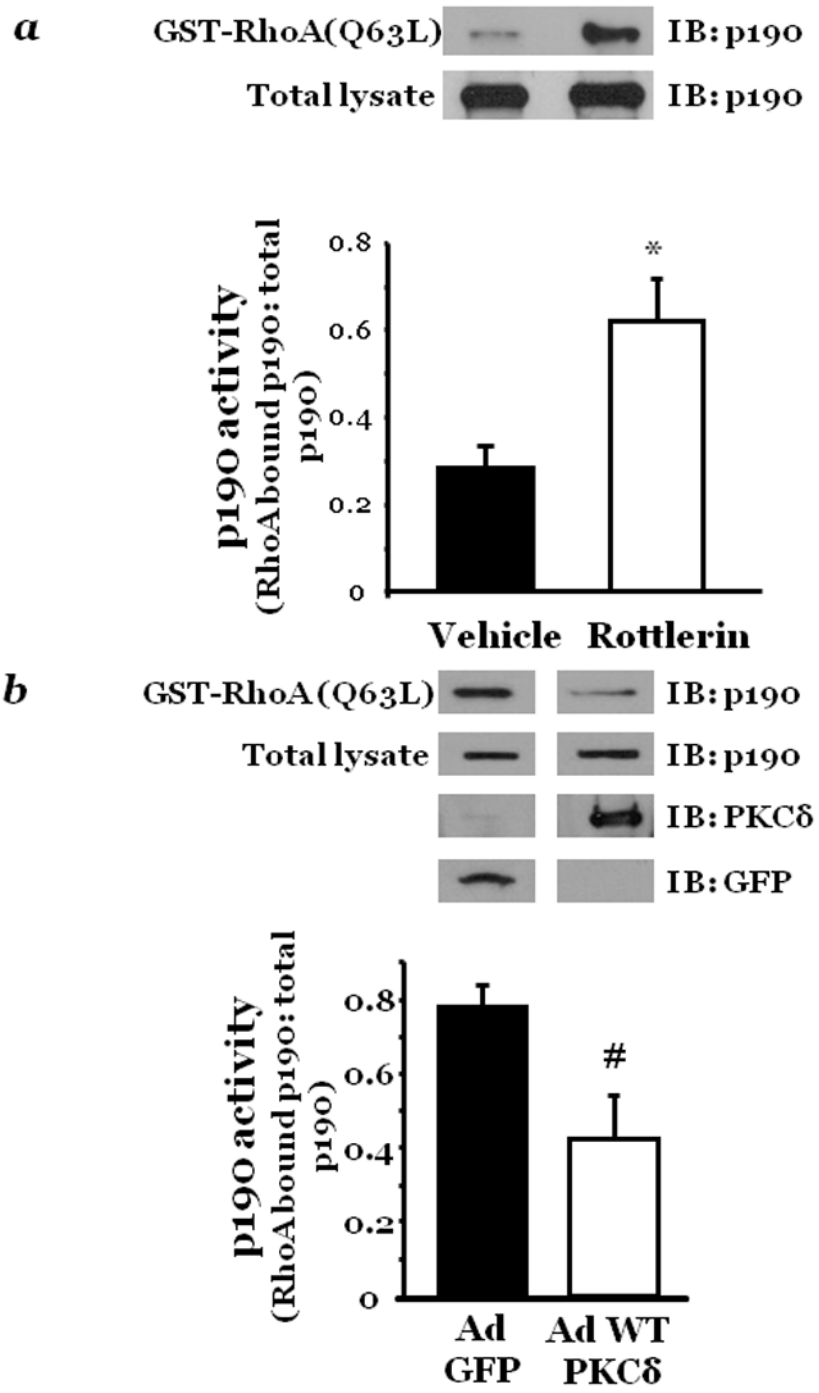


Figure 3. PKCδ activity inversely affects p190 activity

Confluent lung microvascular endothelial cells (LMVEC) were treated with vehicle (DMSO) or 10μM rottlerin for 30 minutes (*panel a*) or infected with adenoviral vectors encoding GFP or wild-type PKCδ cDNA (*panel b*). Cells were harvested and p190 activity determined as the level of p190 bound to GST-fused constitutively actvated RhoA. The level of active p190 relative to total p190 was determined by densitometry. In *panel b*, GFP and PKCδ overexpression was confirmed in the transfected endothelial cells by immunoblot analysis. Data are presented as the mean±SE. *Panel a*, n=4; $^{*}p<0.05$ vs. vehicle. *Panel b*,

n=7, #p<0.05 vs. GFP. *Panels a* and *b*: Reprinted from Fordjour, A.K. and Harrington, E.O. 2009. PKCδ influences p190 phosphorylation and activity: Events independent of PKCδ-mediated regulation of endothelial cell stress fiber and focal adhesion formation and barrier function. *Biochimica et Biophysica Acta*, 1790:1179–1190.

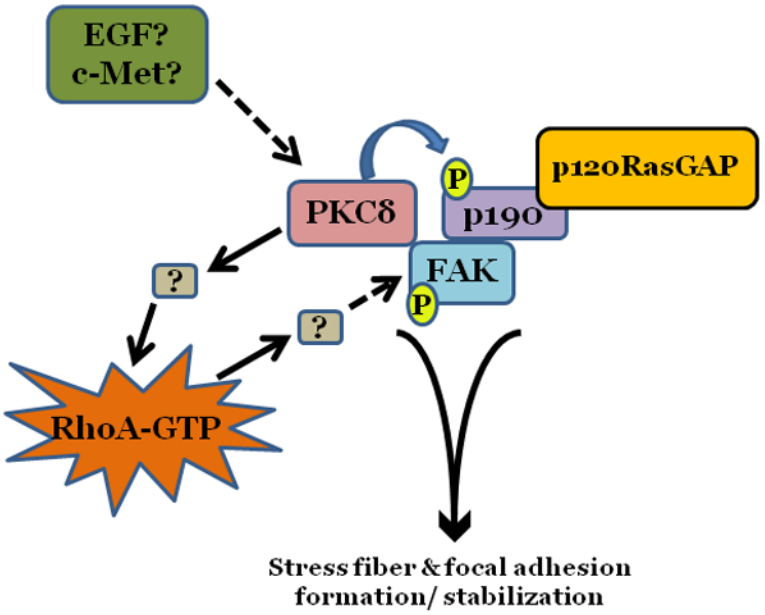


Figure 4. Model of potential cross talk between PKCδ, p190, and FAK

Under basal conditions, PKCδ functions to maintain endothelial barrier function through maintenance of a static level of RhoA activation and stimulation of FAK autophosphorylation. This allows for focal adhesion stabilization and organization of actin stress fibers. PKCδ-mediated activation of RhoA appears to occur independently of its effects on p190 phosphorylation.