Signaling pathways involved in OxPAPC-induced pulmonary endothelial barrier protection

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Abstract

Increased tissue or serum levels of oxidized phospholipids have been detected in a variety of chronic and acute pathological conditions such as hyperlipidemia, atherosclerosis, heart attack, cell apoptosis, acute inflammation and injury. We have recently described signaling cascades activated by oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC) in the human pulmonary artery endothelial cells (EC) and reported potent barrier-protective effects of OxPAPC, which were mediated by small GTPases Rac and Cdc42. In this study we have further characterized signal transduction pathways involved in the OxPAPC-mediated endothelial barrier protection. Inhibitors of small GTPases, protein kinase A (PKA), protein kinase C (PKC), Src family kinases and general inhibitors of tyrosine kinases attenuated OxPAPC-induced barrier-protective response and EC cytoskeletal remodeling. In contrast, small GTPase Rho, Rho kinase, Erk-1,2 MAP kinase and p38 MAP kinase and PI3-kinase were not involved in the barrier-protective effects of OxPAPC. Inhibitors of PKA, PKC, tyrosine kinases and small GTPase inhibitor toxin B suppressed OxPAPC-induced Rac activation and decreased phosphorylation of focal adhesion kinase (FAK) and paxillin. Barrier-protective effects of OxPAPC were not reproduced by platelet activating factor (PAF), which at high concentrations induced barrier dysfunction, but were partially attenuated by PAF receptor antagonist A85783. These results demonstrate for the first time upstream signaling cascades involved in the OxPAPC-induced Rac activation, cytoskeletal remodeling and barrier regulation and suggest PAF receptor-independent mechanisms of OxPAPC-mediated endothelial barrier protection.

Keywords

PKC; PKA; Rac; FAK; paxillin; phosphorylation; endothelium; permeability; actin; cytoskeleton

INTRODUCTION

The vascular endothelial monolayer forms a semi-selective permeable barrier between the blood and the interstitial space, which regulates macromolecule transport and leukocyte trafficking through the vessel wall. A number of agonists including phospholipid products (Zhang, Magnusson et al. 1997;Huang, Subbaiah et al. 2005) and bioactive peptides (Garcia, Siflinger-Birnboim et al. 1986;van Nieuw Amerongen, Draijer et al. 1998;Petrache, Verin et al. 2001;Moy, Blackwell et al. 2002;Birukova, Adyshev et al. 2005) increase vascular endothelial permeability, which my lead to alveolar flooding and pulmonary edema. However, much smaller number of bioactive molecules capable of enhancing endothelial barrier properties have been reported so far (Garcia, Liu et al. 2001;Liu, Schaphorst et al. 2002;Vouret-Craviari, Bourcier et al. 2002;Birukov, Bochkov et al. 2004;Kolosova, Mirzapoiazova et al. 2005), and signaling mechanisms underlying these barrier protective effects have been a major focus of ongoing studies (Lee, Lee et al. 2000;Garcia, Liu et al. 2001;Vouret-Craviari, Bourcier et al. 2002;Birukov, Leitinger et al. 2004;Dudek, Jacobson et al. 2004;Kolosova, Mirzapoiazova et al. 2005;Mehta, Konstantoulaki et al. 2005;Singleton, Dudek et al. 2005).

Phosphocholines represent a major group of cell membrane structural phospholipids. Enhanced lipid peroxidation leading to formation of oxidized phospholipids was observed in acute lung injury syndromes such as ARDS, ventilator-induced lung injury, and asthma (Chabot, Mitchell et al. 1998;Wood, Gibson et al. 2003). An increased release of membrane vesicles containing oxidized phospholipids has been detected as a result of tissue injury and apoptosis (Holme, Solum et al. 1994;Huber, Vales et al. 2002), and such membrane vesicles were also observed in patients with acute lung injury, cardiac ischemia, acute coronary syndrome and in pathological settings with platelet activation (Holme, Solum et al. 1994;Mallat, Benamer et al. 2000).

We have recently described that oxidized phospholipids derived from oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (OxPAPC) exhibited potent barrier-protective effects towards human pulmonary endothelial monolayers (Birukov, Bochkov et al. 2004). Formation of rigid peripheral cytoskeletal structures is a morphological basis of endothelial barrier enhancement and is regulated by several signaling pathways with small GTPases Rac and Cdc42 playing a major role in the triggering of EC barrier-protective response (Lum and Malik 1996;Dudek and Garcia 2001;Garcia, Liu et al. 2001;Birukov, Bochkov et al. 2004;Kouklis, Konstantoulaki et al. 2004). Using molecular approaches including ectopic expression of mutant small GTPases and siRNA-based small GTPase protein depletion, we linked barrier-protective effects of OxPAPC to Rac/Cdc42-mediated enhancement of peripheral actin cytoskeleton (Birukov, Bochkov et al. 2004).

In various types of endothelial cells, OxPAPC also activates small GTPase R-Ras (Cole, Subbanagounder et al. 2003), stimulates protein kinase C, protein kinase A, protein tyrosine kinases, and induces the Raf / MEK1,2 / Erk-1,2 and JNK MAP kinase signaling cascades (Cole, Subbanagounder et al. 2003;Birukov, Bochkov et al. 2004;Birukov, Leitinger et al. 2004), as well as transcriptional STAT3 pathway (Yeh, Gharavi et al. 2004). Importantly, these effects are not reproduced by non-oxidized PAPC (Birukov, Leitinger et al. 2004). Activation of PKC and the MEK/Erk-1,2 MAP kinase cascade by OxPAPC triggers expression of early growth response factor 1 (Egr-1) and tissue factor in HUVEC monolayers (Bochkov, Mechtcheriakova et al. 2002). OxPAPC-induced PKA activation is involved in the OxPAPC-induced heme oxygenase-1 induction (Kronke, Bochkov et al. 2003) and deposition of CS-1 fibronectin on apical $\alpha_v\beta_1$ integrin critical for monocyte binding to arterial endothelium from systemic circulation (Cole, Subbanagounder et al. 2003). In addition, OxPAPC induces activation of tyrosine kinases including Src kinase and focal adhesion kinase (FAK) (Birukov, Leitinger et al. 2004;Yeh, Gharavi et al. 2004), resulting in activation of focal adhesion

complexes and cytoskeletal remodeling. Thus, a number of signaling pathways induced by OxPAPC have been reported so far. However, specific protein kinase pathways involved in OxPAPC-mediated EC barrier-protective response have not been yet characterized.

A small proportion of phospholipids contain *sn*-1 residues attached to glycerol by an ether bond. Upon oxidative fragmentation of *sn*-2 unsaturated fatty acid residues, these alkyl-oxidized phospholipids become agonists for the platelet-activating factor (PAF) receptor (Marathe, Davies et al. 1999). These "PAF-like lipids" are formed *in vivo* and induce many effects typical of PAF (Marathe, Prescott et al. 2001). However, potential involvement of PAF receptor in OxPAPC-mediated EC permeability responses has not been yet studied.

In this study, we characterized protein kinase signaling cascades involved in barrier-protective response to OxPAPC, studied relations between protein kinase A, protein kinase C, Src kinase and Rac GTPase in OxPAPC-induced endothelial barrier regulation and examined involvement of PAF receptor in the OxPAPC-mediated EC barrier regulation.

MATERIALS AND METHODS

Reagents and cell culture

All biochemical reagents including PAF, A85783, and PAPC were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. PAPC was oxidized by exposure of dry lipid to air for 72 hours. The extent of oxidation was monitored by positive ion electrospray mass spectrometry as described previously (Watson, Leitinger et al. 1997). Each batch of oxidized phospholipids was standardized by thin layer chromatography. In addition, quality control of the OxPAPC composition in each batch was also performed by positive ion electrospray mass spectrometry and judged by a standard pattern of characteristic peaks with *m/z* 616.4; 632.4; 810.5; 828.5; corresponding to major oxygenated and fragmented products of PAPC oxidation. Specific effects of oxygenated (PECPC, PEIPC) and fragmented (POVPC, PGPC, lyso-PC) components present in standard OxPAPC preparations have been characterized previously (see (Bochkov and Leitinger 2003;Birukov 2006;Bochkov, Leitinger et al. 2006) for review). PAPC and OxPAPC preparations were shown negative for endotoxin by the limulus amebocyte assay performed after PAPC oxidation prior to *in vivo* and *in vitro* experiments (BioWhittaker, Frederick, MD). All reagents used for immunofluorescent staining were purchased from Molecular Probes (Eugene, OR). Toxin B, genistein, PP2, Y27632, SB 203580, LY294002 were purchased from Calbiochem (La Jolla, CA), U0126 and cell permeant myristoylated PKA and PKC inhibitory peptides were purchased from Promega (Madison, WI), phospho-FAK (Tyr[576/577]), FAK, phospho-Src (Tyr[416]), Src, and p-paxillin (Tyr[118]) antibodies were obtained from Cell Signaling (Beverly, MA), phospho-tyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY), paxillin antibody was purchased from BD Transduction Laboratories (San Diego, CA). Human pulmonary artery endothelial cells were obtained from Clonetics, BioWhittaker Inc. (Frederick, MD), maintained according to vendor's protocol, and used at passages 6–10.

Measurement of transendothelial electrical resistance

The cellular barrier properties were measured according to the method described elsewhere (Tiruppathi, Malik et al. 1992). The total transendothelial electrical resistance (TER) was measured dynamically across the monolayer using an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) and was determined by the combined resistance between the basal surface of the cell and the electrode, reflective of focal adhesion, and the resistance between the cells, as described previously (Birukov, Bochkov et al. 2004;Birukova, Smurova et al. 2004).

Immunofluorescent staining

EC monolayers were fixed after agonist treatment in 3.7% formaldehyde solution in PBS for 10 min at 4° C, washed three times with PBS, permeabilized with 0.2% triton X-100 in PBS-Tween (PBST) for 30 min at room temperature, and blocked with 2% BSA in PBST for 30 min. Incubation with antibody of interest was performed in 2% BSA–PBST for 1 hr at room temperature followed by staining with Texas Red-conjugated phalloidin (Molecular Probes, Eugene, OR) to visualize actin filaments. After immunostaining, the glass slides were analyzed using Nikon video-imaging system (Nikon Instech Co., Tokyo, Japan). The images were recorded and processed using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) software.

Rac activation assay

Activation of Rac GTPase in pulmonary EC culture were analyzed using Rac activation assay kit available from Upstate Biotechnology (Lake Placid, NY) according to manufacturer's protocol, as previously described (Birukova, Smurova et al. 2004;Shikata, Rios et al. 2005).

Western immunoblotting

Protein extracts were separated by SDS-PAGE on 10% gels, transferred to nitrocellulose membrane (100 V for 1 h), and probed with specific antibodies. Immunoreactive proteins were detected using enhanced a chemiluminescent detection system (ECL) according to the manufacturer's protocol (Amersham, Little Chalfont, UK). The relative intensities of the protein in the bands were quantified by scanning densitometry.

Statistical Analysis

Results are expressed as means $\pm$ SD of three to five independent experiments. Stimulated samples were compared with controls by unpaired Student's *t*-test. For multiple-group comparisons, one-way analysis of variance (ANOVA) followed by the post hoc Fisher's test were used. $P < 0.05$ was considered statistically significant.

RESULTS

Involvement of cellular signaling pathways in barrier enhancement induced by oxidized phospholipids

We have previously shown involvement of Rac and Cdc42 in the OxPAPC-induced EC barrier protection (Birukov, Bochkov et al. 2004). Furthermore, we have described OxPAPC-induced activation of protein kinase A, protein kinase C, protein tyrosine phosphorylation, FAK and paxillin phosphorylation, as well as activation of Erk-1,2, p38 and JNK MAP kinase cascades in the human pulmonary EC (Birukov, Leitinger et al. 2004). In these experiments we studied OxPAPC-induced signaling pathways, which mediate OxPAPC-induced EC barrier protection. We performed inhibitory analysis and examined effects of cell-permeable peptide inhibitors of PKA (20 μM), PKC (20 μM) (Promega, Madison, WI), broad range tyrosine kinase inhibitor genistein (100 μM), inhibitor of Src family tyrosine kinases PP2 (2 μM), MEK inhibitor U0126 (5 μM), p38 MAPK inhibitor SB203580 (20μM), and PI3 kinase inhibitor LY294002 (25 μM) on OxPAPC-induced increases in transendothelial resistance across EC monolayers. Because Rac and Cdc42, but not Rho mediate OxPAPC- induced barrier enhancement (Birukov, Bochkov et al. 2004), in this study we used a general Rac, Rho and Cdc42 inhibitor from *C. difficile* toxin B (20 ng/ml) and Rho kinase inhibitor Y27632 (5 μM) as controls. EC monolayers pre-treated with specific inhibitors were stimulated with OxPAPC (20 μg/ml), and TER changes monitored during 2 hours were expressed as percent of maximal barrier-protective response induced by OxPAPC. EC pretreatment with toxin B or inhibitors of PKA, PKC, or tyrosine phosphorylation significantly attenuated OxPAPC-induced increases in TER (Figure 1). In contrast, inhibition of Rho- and Erk-1,2-dependent pathways, p38 MAPK, and PI3 kinase

were without effect. To further evaluate an involvement of specific signaling pathways in OxPAPC-mediated cytoskeletal changes associated with EC barrier protective response, pulmonary EC were pretreated with pharmacological inhibitors followed by OxPAPC stimulation (20 μg/ml, 30 min), and F-actin remodeling was analyzed by immunofluorescent staining with Texas Red conjugated phalloidin (Figure 2). Consistent with previous studies (Birukov, Bochkov et al. 2004), OxPAPC stimulation caused disappearance of central actin stress fibers and pronounced accumulation of F-actin at the cell periphery (shown by arrows), as compared to quiescent EC monolayers. Remarkably, EC pre-incubation with toxin B or inhibitors of PKA, PKC and tyrosine kinase not only abolished disappearance of central actin stress fibers, but significantly attenuated F-actin accumulation at the areas of intercellular junctions. In contrast, inhibition of Erk-1,2 and p38 MAPK kinases, Rho-kinase and PI3-kinase did not affect the OxPAPC-induced cytoskeletal remodeling consistent with the lack of effects on OxPAPC-induced TER increases. These results strongly suggest an essential role of tyrosine phosphorylation, PKA and PKC-dependent signaling in OxPAPC-mediated barrier enhancement.

Involvement of PKA and PKC-dependent pathways in OxPAPC-induced Rac activation

Activation of small GTPase Rac has been previously described as a leading mechanism of barrier protection in response to various stimuli including OxPAPC, sphingosine 1-phosphate, hepatocyte growth factor (HGF), and shear stress (Garcia, Liu et al. 2001;Birukov, Birukova et al. 2002;Liu, Schaphorst et al. 2002;Birukov, Bochkov et al. 2004;Shikata, Rios et al. 2005). In the next studies we investigated the roles of protein kinases A and C and tyrosine kinases in the regulation of OxPAPC-induced Rac activation. HPAEC preincubated with cell permeable peptide inhibitors of PKA and PKC, or pretreated with general tyrosine kinase inhibitor genistein for 30 min were next stimulated with OxPAPC. Toxin B pretreatment served as a positive control for Rac inhibition. Inhibition of PKA, PKC and protein tyrosine phosphorylation significantly attenuated OxPAPC-induced Rac activation (Figure 3).

Effect of PKA and PKC on OxPAPC-induced protein tyrosine phosphorylation

Tyrosine phosphorylation of focal adhesion and cytoskeletal proteins is essential for regulation of endothelial barrier (Mehta, Tiruppathi et al. 2002;Shikata, Birukov et al. 2003;van Nieuw Amerongen, Natarajan et al. 2004). Previous study showed increased FAK and paxillin phosphorylation in response to OxPAPC (Birukov, Leitinger et al. 2004). We next examined involvement of PKA and PKC signaling in the OxPAPC-induced protein tyrosine phosphorylation. EC preincubated for 30 min with toxin B (20 ng/ml) or cell permeable peptide inhibitors of PKA (20 μM) and PKC (20 μM) were treated with OxPAPC (20 μg/ml, 30 min). Genistein (100 μM, 1 hr) was used as a broad inhibitor of tyrosine kinases. Obtained cell lysates were probed in immunoblot with site-specific phosphotyrosine antibodies to Src-Tyr[416], FAK-Tyr[576/577] (Figure 4A,B), and paxillin-Tyr[118] (Figure 4C). Inhibition of PKA and PKC, as well as inhibition of small GTPases by toxin B significantly reduced site-specific tyrosine phosphorylation of Src, FAK and paxillin in response to OxPAPC challenge.

Role of PAF receptor-dependent signaling in the regulation of OxPAPC-mediated barrier protection

Because selected biological effects of OxPAPC can be partially inhibited by platelet activating factor (PAF) receptor antagonists (Leitinger, Watson et al. 1997;Subbanagounder, Leitinger et al. 1999;Kadl, Huber et al. 2002), we next tested effects of PAF in the model of pulmonary EC permeability and examined potential involvement of PAF receptor in OxPAPC-mediated barrier-protective responses, cytoskeletal remodeling, and protein phosphorylation. In contrast to OxPAPC, PAF stimulation did not induce TER increases in HPAEC monolayers (Figure 5A) at hormonal concentrations (10 – 50 nM), whereas at high non-physiological

concentrations (5 – 100 µg/ml), PAF caused dose-dependent TER decline reflective of pronounced EC barrier dysfunction (Figure 5A), which was not affected by PAF receptor antagonist A85783 (5 µM, 1 hr) (Figure 5B,D). In contrast to effects on PAF responses, A85783 partially attenuated OxPAPC-induced increase in transendothelial resistance (Figure 5C,D). Next, we investigated effects of PAF receptor antagonist A85783 on OxPAPC-induced cytoskeletal remodeling. A85783 alone did not change EC morphology, but partially attenuated OxPAPC-induced peripheral F-actin accumulation (Figure 5E). Interestingly, PAF receptor antagonist also partially decreased OxPAPC-induced paxillin phosphorylation and general protein tyrosine phosphorylation.

Discussion

The results of this study demonstrate for the first time the role for PKA and PKC as upstream regulators of Rac activity in response to OxPAPC stimulation and indicate that Rac activity was required for OxPAPC-induced tyrosine phosphorylation of Src, FAK, paxillin, which was essential for EC cytoskeletal remodeling and barrier protective response.

Previous studies have shown that Src kinase inhibition abolished OxPAPC-induced phosphorylation of FAK and its substrate paxillin, a focal adhesion protein, which functions as structural and adaptor protein (Birukov, Leitinger et al. 2004). This study shows that inhibitors of PKA and PKC abolished OxPAPC-induced Rac activation, whereas MEK/Erk-1,2 and p38 MAP kinase inhibitors were without effect. In turn, downregulation of small GTPases by cell pretreatment with toxin B attenuates phosphorylation of Src, FAK and paxillin. Since OxPAPC does not activate Rho, these results suggest involvement of Rac-dependent mechanism in OxPAPC-induced tyrosine phosphorylation of Src, FAK, and paxillin, which may be an important part of coordinated focal adhesion and cytoskeletal remodeling associated with barrier-protective effects of OxPAPC. Rac-dependent mechanisms of tyrosine kinase activation are not well understood, but may involve induction of NADPH oxidase, production of reactive oxygen species (ROS) and ROS-induced stimulation of tyrosine phosphorylation (Rhee, Chang et al. 2003).

Regulation of Rac activation by PKC and PKA may involve modulation of specific guanosine nucleotide exchange factors (GEFs). Tiam1 is a Rac-specific GEF regulated by diverse mechanisms including phosphorylation by Ca^{2+}/calmodulin–dependent kinase II (CaMKII) or related kinases, interaction with phosphatidylinositol-3-kinase (PI3K) product PtdIns(3,4,5)P_3, binding to cell surface molecule CD44 or cytoskeletal protein ankyrin, as well as by direct binding to activated Ras (Zheng 2001). Tiam1 activity may be also regulated by tyrosine phosphorylation (Servitja, Marinissen et al. 2003). Src-dependent phosphorylation of Tiam1 has been recently implicated in the Rac activation induced by another barrier-protective agonist, sphingosine 1-phosphate (Gonzalez, Kou et al. 2006). Thus, control of Tiam1 activity by PKC-, tyrosine kinase-, and PKA-dependent mechanisms may be a potential mechanism of OxPAPC-induced Rac regulation. Ongoing studies from our lab are addressing these important questions.

Upon oxidative fragmentation of *sn*-2 unsaturated fatty acid residues, OxPL may become agonists for the platelet-activating factor (PAF) receptor (Marathe, Davies et al. 1999). These "PAF-like lipids" are formed *in vivo* and induce many effects typical of PAF (Marathe, Prescott et al. 2001). In the case of fragmented acyl-oxidized phospholipids, however, many biological actions can not be explained solely by the ability to stimulate PAF receptors, because many effects of these acyl-oxidized phospholipids are not reproduced by PAF (Marathe, Davies et al. 1999,Leitinger, Watson et al. 1997;Subbanagounder, Leitinger et al. 1999;Subbanagounder, Watson et al. 2000). In this study we examined potential involvement of PAF receptor signaling in the barrier-protective effects of OxPAPC. Our results indicate that OxPAPC-induced barrier-

protective responses were not mimicked by PAF, which did not decrease endothelial permeability at hormonal concentrations (0.1–1.0 μg/ml), but instead, induced dramatic barrier dysfunction and cytotoxic effects at non-physiologically high concentrations (20– 50 μg/ml), possibly by disrupting endothelial barrier in a nonspecific fashion or by acting as detergent. These effects of high PAF concentrations were not affected by PAF receptor antagonist A85783 (data not shown). However, PAF receptor antagonist partially attenuated barrier-protective effects of OxPAPC. These results suggest an inhibitory effect of A85783 on a putative OxPAPC receptor involved in EC barrier protective response, which is distinct from PAF receptor. Our results also do not exclude a possibility that several receptors may bind different components of OxPAPC and trigger different pathways of OxPAPC-mediated signal transduction (Leitinger, Tyner et al. 1999). For example, recent report (Cole, Subbanagounder et al. 2003) proposes a hypothetical G protein-coupled receptor involved in activation of CS-1 fibronectin production by OxPAPC. Other study indicates that epoxyisoprostane-containing products of PAPC oxidation may engage prostaglandin E2 (EP2) receptor and elevate intracellular cAMP levels (Li, Mouillesseaux et al. 2006), which may promote endothelial barrier function (Moy A. B. 1998;Birukova, Liu et al. 2004). However, EP2-mediated effects can not be blocked by PAF receptor antagonists suggesting additional A85783-sensitive receptors mediating barrier protective effects of OxPAPC. Importantly, putative OxPAPC receptor mediating barrier protective response appears to be specifically activated by epoxycyclopenthenone-containing products of PAPC oxidation such as PEIPC and PECPC, but not by fragmented OxPAPC derivates, such as lyso-phosphocholine, POVPC or PGPC, which exhibit barrier-disruptive properties (Birukov, Bochkov et al. 2004).

In summary, this study defined signaling pathways involved in OxPAPC-induced EC barrier protective response and described PKA, PKC and tyrosine kinases as upstream activators of Rac/Cdc42 pathway of OxPAPC-induced signaling in focal adhesions, cytoskeletal remodeling and barrier protection. Our results indicate that OxPAPC-induced barrier-protective responses are not mediated by PAF receptor. Based on the results of this study and previous works, we propose a hypothetical scheme of EC barrier regulation by OxPAPC (Figure 6). Interaction of oxygenated PAPC products such as PEIPC and PECPC with putative receptor stimulates protein tyrosine kinases, PKA and PKC, which activate Rac specific GEF (s) and promote GDP/GTP exchange in the nucleotide binding center of the Rac leading to its activation. Other effects of tyrosine kinase activation include phosphorylation of focal adhesion proteins and focal adhesion redistribution essential for anchoring of peripheral actin cytoskeleton and enhancement of EC barrier (Shikata, Birukov et al. 2003). Additional effects of PKC activation include stimulation of Erk-1,2 MAP kinase cascade (Birukov, Leitinger et al. 2004), which is however not involved in OxPAPC-mediated EC cytoskeletal remodeling and barrier protective responses (Figures 1–3). Activated Rac interacts with downstream cytoskeletal and cell adhesion effectors and promotes cytoskeletal remodeling and EC barrier enhancement. In addition, activation of Rac stimulates Src kinase and leads to phosphorylation of FAK and paxillin. These two signaling focal adhesion proteins are essential for initiation of focal adhesion remodeling observed in response to OxPAPC stimulation.

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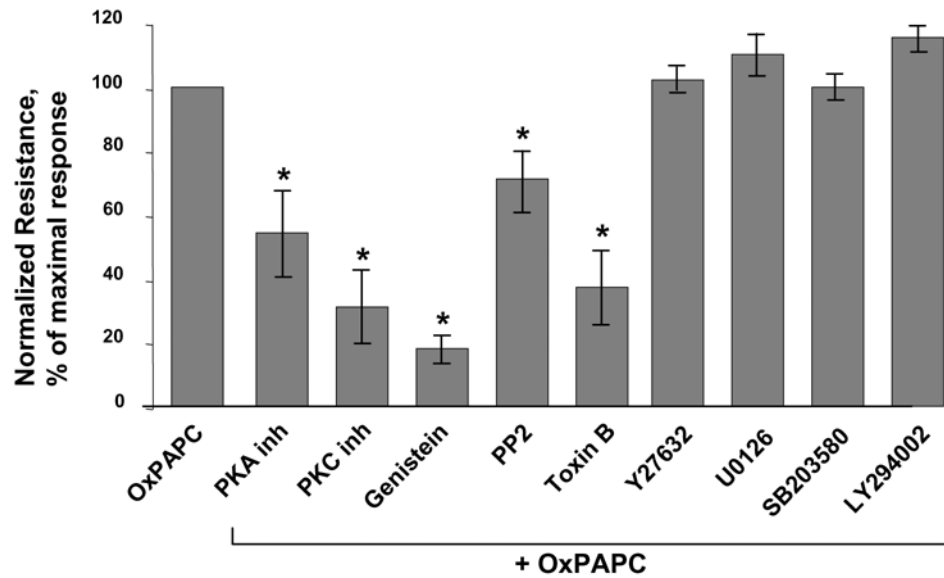
Figure 1. Inhibitory analysis of signaling pathways involved in OxPAPC-mediated increase in EC resistance
Transendothelial resistance was monitored across confluent endothelial monolayers pretreated with vehicle, PKA inhibitor (20 μM), PKC inhibitor (20 μM), genistein (100 μM), PP2 (2 μM), toxin B (20 ng/ml), Y27632 (5 μM), U0125 (5 μM), SB 203580 (20 μM), or LY294002 (25 μM) for 30 min followed by addition of OxPAPC (20 μg/ml). Bar graphs represent measurements of TER after 30 min of OxPAPC stimulation. Shown are results of three to seven independent experiments represented as mean ± SE. *p<0.01 vs OxPAPC alone.
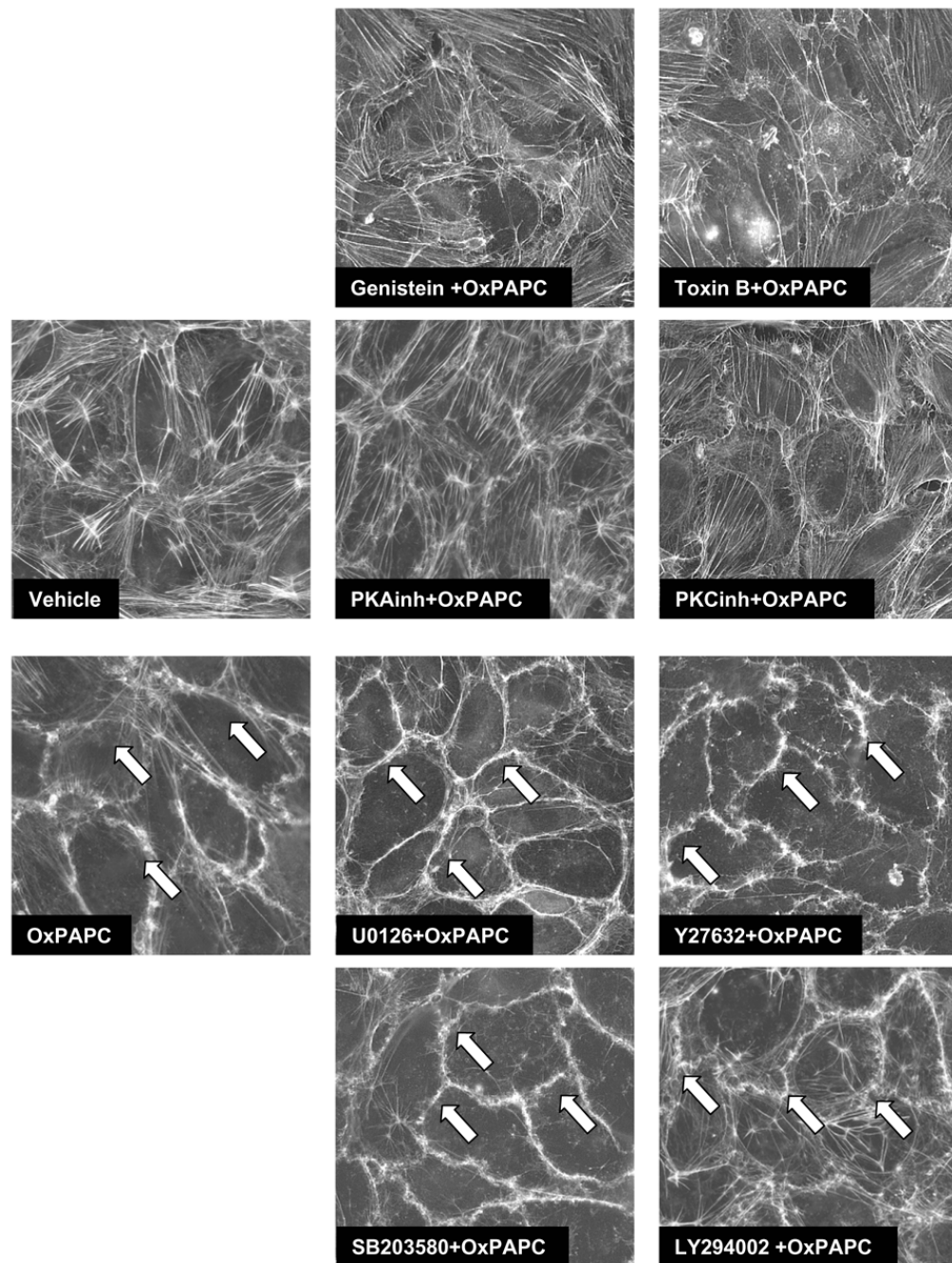
Figure 2. Analysis of signaling pathways involved in OxPAPC-induced cytoskeletal remodeling
EC grown on glass coverslips were preincubated with genistein (100 μM), small GTPase
inhibitor toxin B (20 ng/ml), PKA inhibitor (20 μM), PKC inhibitor (20 μM), Rho kinase
inhibitor Y27632 (5 μM), MEK/Erk1,2 inhibitor U0125 (5 μM), p38 MAP kinase inhibitor
SB203580 (20 μM), or PI3-kinase inhibitor LY294002 (25 μM) for 30 min followed by addition
of OxPAPC (20 μg/ml) for 30 min. Cytoskeletal remodeling was assessed by
immunofluorescent staining for F-actin with Texas Red phalloidin. Results are representative
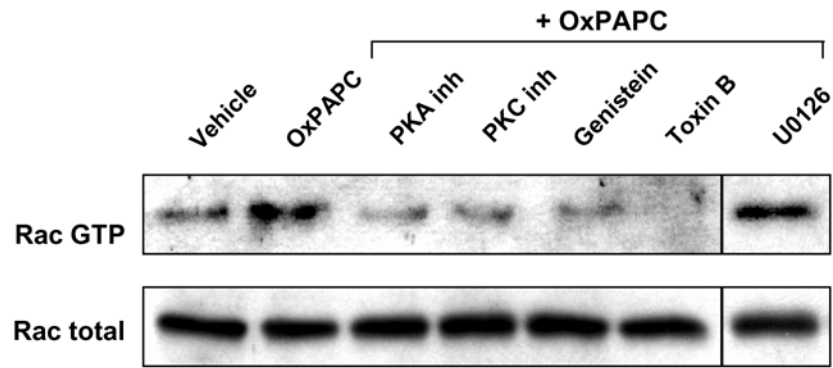of three independent experiments.

Figure 3. Effect of PKA and PKC inhibition on OxPAPC-induced Rac activation
HPAEC were preincubated with PKA inhibitor (20 μM), PKC inhibitor (20 μM), toxin B (20 ng/ml), genistein (100 μM) or U0125 (5 μM) for 30 min followed by addition of OxPAPC (20 μg/ml) for 5 min. Rac activation was analyzed as described in Methods section. Results are representative of three independent experiments.
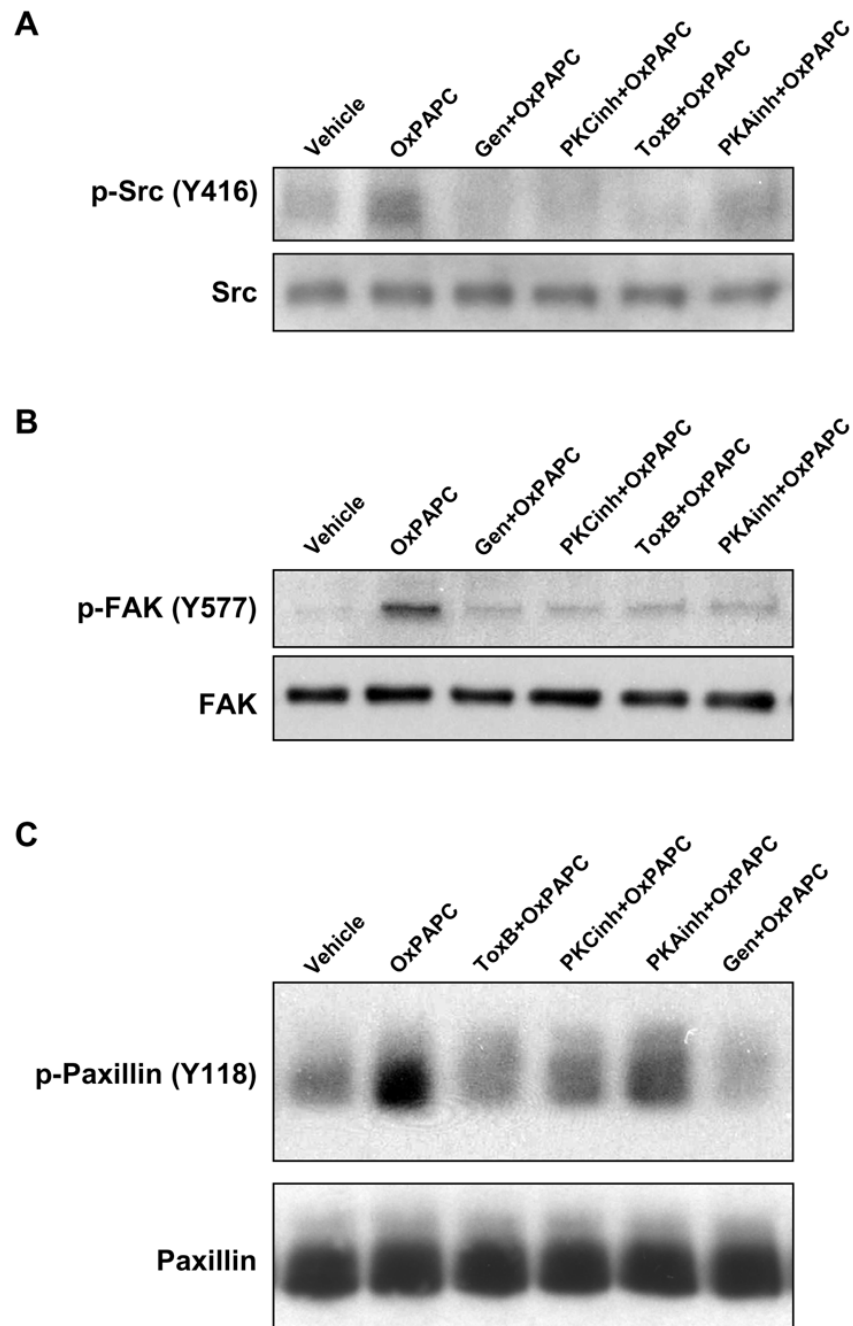
Figure 4. Effect of PKA and PKC on OxPAPC-induced protein tyrosine phosphorylation
EC were pretreated with genistein (100 μM), PKA inhibitor (20 μM), PKC inhibitor (20 μM), or toxin B (20 ng/ml) for 30 min followed by stimulation with OxPAPC (20 μg/ml) for 30 min. Phosphorylation of specific proteins was detected by western blot with specific antibodies to phospho-Src (Tyr[416]) **(panel A)**, phospho-FAK (Tyr[576/577]) **(panel B)** and phospho-paxillin (Tyr[118]) **(panel C)**. Equal protein loadings were confirmed by reprobing the membranes with Src, FAK or paxillin antibodies, respectively. Shown are representative results of three independent experiments.
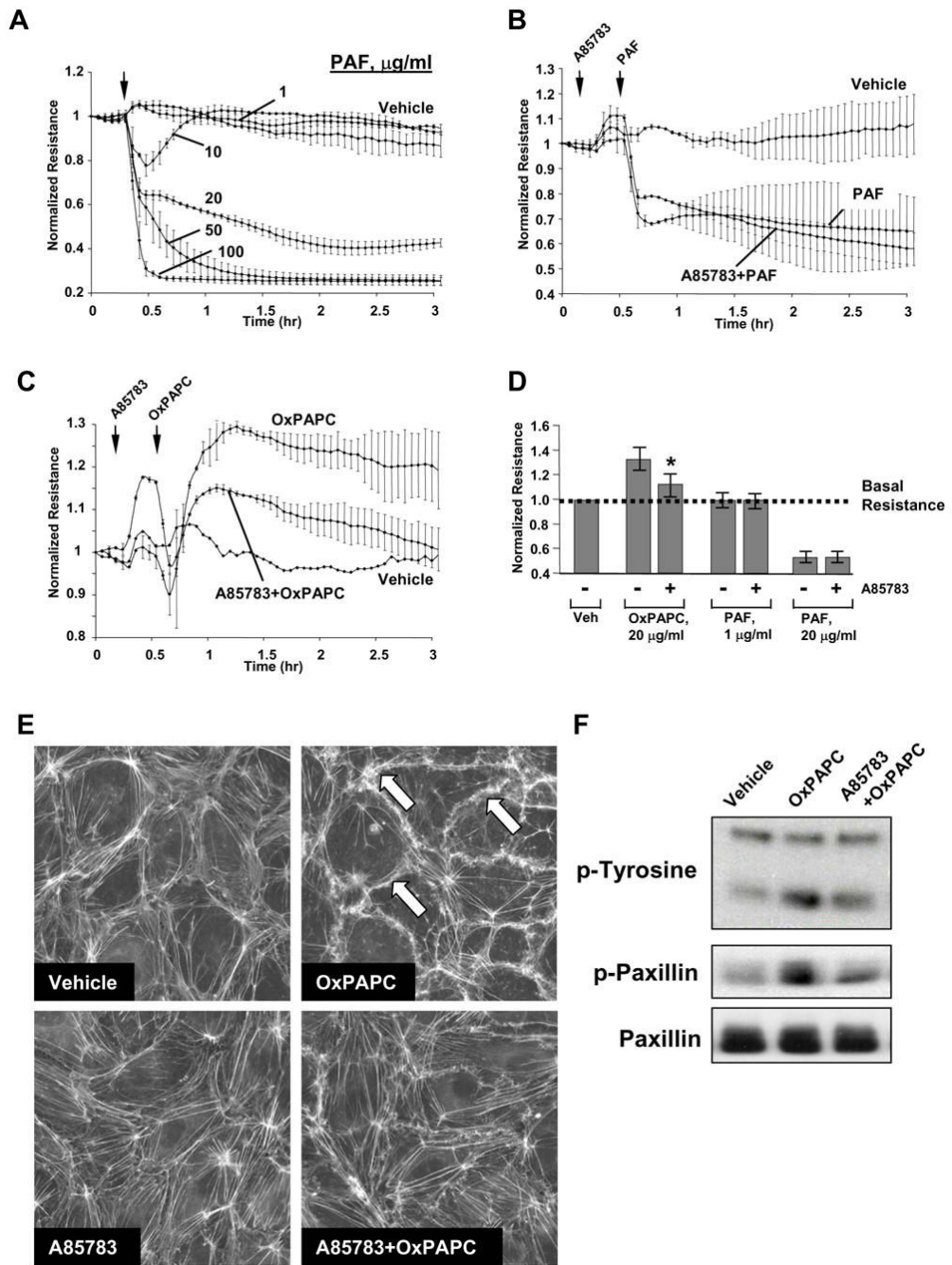
Figure 5. Analysis of potential involvement of PAF receptor in OxPAPC-induced signaling and endothelial barrier protection

Panels A – D: TER measurements in EC monolayers. Cells grown on gold microelectrodes were stimulated with PAF (1 μg/ml, 5 μg/ml, 20 μg/ml, 50 μg/ml, 100 μg/ml) **(panel A)**; or were preincubated with PAF receptor antagonist A85783 (5 μM, 30 min) followed by stimulation with 20 μg/ml of PAF **(panel B)** or OxPAPC **(panel C)**. Effects of PAF receptor antagonist on PAF- and OxPAPC-mediated permeability changes are summarized in **panel D**. Shown are pooled data of three independent experiments represented as mean + SD. *p<0.01 vs OxPAPC alone. **Panel E**: EC were preincubated with A85783 (5 μM, 30 min) followed by stimulation with OxPAPC (20 μg/ml, 30 min) and immunofluorescent analysis of F-actin

remodeling by staining with Texas Red phalloidin. **Panel F:** EC preincubated with vehicle or A85783 (5 μM, 30 min) were treated with OxPAPC, and total protein tyrosine phosphorylation and site-specific paxillin phosphorylation was detected by immunoblotting with phospho-tyrosine or phospho-paxillin (Tyr[118]) antibodies. Equal protein loading was confirmed by reprobing the membranes with paxillin antibody.
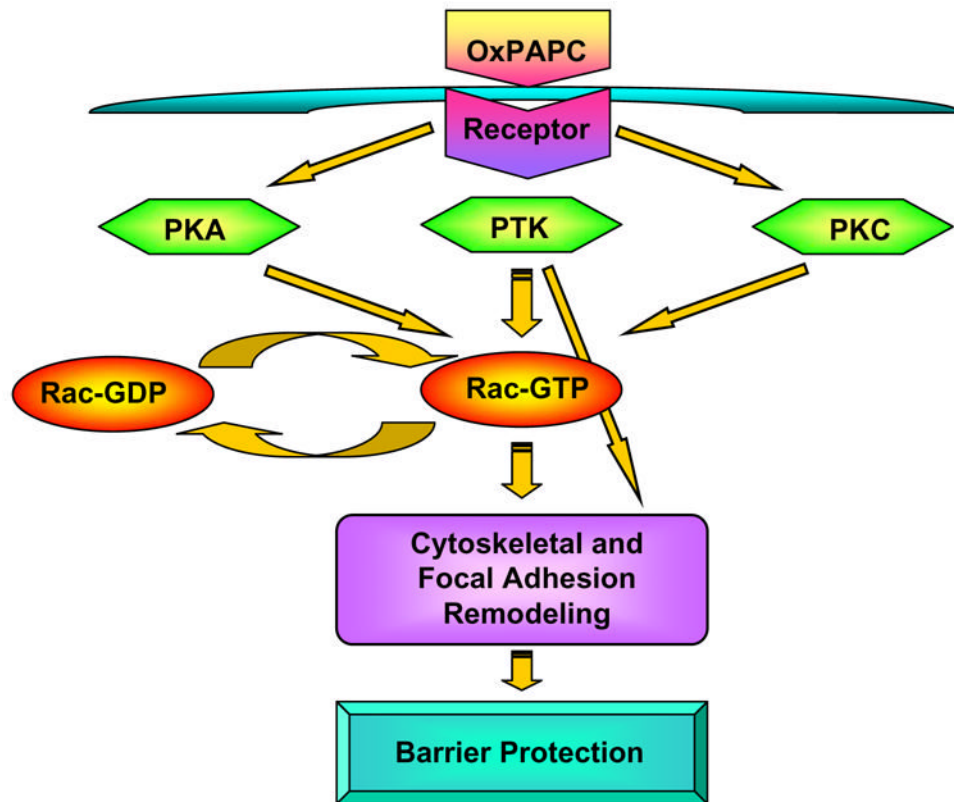
Figure 6. Proposed mechanism of OxPAPC-mediated activation of Rac and regulation of cytoskeletal rearrangement and barrier function

OxPAPC treatment stimulates PKA, PKC and protein tyrosine kinases. PKA, PKC and tyrosine kinases promote Rac activation via stimulation of Rac specific GEF(s). Activated Rac interacts with downstream cytoskeletal and cell adhesion effectors and promotes cytoskeletal remodeling and EC barrier enhancement. In addition, activation of Rac stimulates Src kinase and leads to phosphorylation of FAK and paxillin, which may further promote assembly of new focal adhesion protein complexes and attachment of newly formed actin filaments to the peripherally redistributed focal adhesions. Mechanisms of tyrosine kinase activation upstream of Rac may be also involved in OxPAPC-induced tyrosine phosphorylation of focal adhesion proteins These events result in formation of peripheral actin rim, focal adhesion remodeling and increased EC monolayer barrier properties.