

# Signal transduction pathways activated in human pulmonary endothelial cells by OxPAPC, a bioactive component of oxidized lipoproteins

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## Abstract

The bioactive component of mildly oxidized low-density lipoproteins, oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (OxPAPC), activates tissue factor expression and monocyte adhesion to endothelial cells (EC) from systemic circulation, but blocks expression of inflammatory adhesion molecules (VCAM, E-selectin) and neutrophil adhesion associated with EC acute inflammatory response to bacterial lipopolysaccharide (LPS). Due to constant exposure to oxygen free radicals, lipids in the injured lung are especially prone to oxidative modification and increased OxPAPC generation. In this study, we focused on OxPAPC-mediated intracellular signaling mechanisms that lead to physiological responses in pulmonary endothelial cells. Our results demonstrate that OxPAPC treatment activated in a time-dependent fashion protein kinase C (PKC), protein kinase A (PKA), Raf/MEK1,2/Erk-1,2 MAP kinase cascade, JNK MAP kinase and transient protein tyrosine phosphorylation in human pulmonary artery endothelial cells (HPAEC), whereas nonoxidized PAPC was without effect. Pharmacological inhibition of PKC and tyrosine kinases blocked activation of Erk-1,2 kinase cascade upstream of Raf. OxPAPC did not affect myosin light chain (MLC) phosphorylation, but increased phosphorylation of cofilin, a molecular regulator of actin polymerization. Finally, OxPAPC induced p60Src-dependent tyrosine phosphorylation of focal adhesion proteins paxillin and FAK. Our results suggest a critical involvement of PKC and tyrosine phosphorylation in OxPAPC-induced activation of Erk-1,2 MAP kinase cascade associated with regulation of specific gene expression, and demonstrate rapid phosphorylation of cytoskeletal proteins, which indicates OxPAPC-induced EC remodeling.

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## Introduction

Oxidized phospholipids are biologically active components of mildly oxidized low-density lipoprotein (LDL), whose role in development of vascular injury and inflammation in systemic circulation is well recognized. Oxidized LDL is implicated in the recruitment of monocytes and foam cell formation (Steinberg et al., 1989), increased expression of matrix metalloproteinases, which is critical for both plaque formation and destabilization (Li et al., 2003), proliferative response of vascular smooth muscle cells (Yang et al., 2001), increased thrombogenic activity

of platelets (Maschberger et al., 2000), and increased endothelial–monocyte interaction (Berliner et al., 1990; Steinberg et al., 1989).

Biologically active oxidized phospholipids derived from oxidation of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (OxPAPC) (Watson et al., 1997) stimulate tissue factor expression (Bochkov et al., 2002b), activate endothelial cells to bind monocytes, but do not cause any neutrophil binding (Bochkov et al., 2002a; Leitinger et al., 1999). In addition, OxPAPC strongly inhibits LPS-mediated induction of neutrophil binding and expression of E-selectin, an adhesion molecule involved in EC inflammatory activation by endotoxin (Bochkov et al., 2002a).

Increased levels of oxidized phospholipids may be associated with acute pathological conditions. Tissue injury, hydrogen peroxide stimulation, and apoptotic processes cause release of membrane vesicles in blood circulation,

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where they become readily oxidized (Freyssinet et al., 1999; Mallat et al., 2000; Patel et al., 1992). Bioactive oxidized components of membrane vesicles or LDL, such as OxPAPC, then exhibit their pathophysiological effects on vascular endothelium (Watson et al., 1997). Indeed, membrane vesicles released by cellular elements were observed in patients with several pathologies including acute coronary syndrome (Mallat et al., 2000), cardiopulmonary bypass (Nieuwland et al., 1997), and in pathological settings with platelet activation (Holme et al., 1994). Furthermore, enhanced lipid peroxidation and formation of oxidized phospholipids in the lung has been demonstrated in patients with diverse lung diseases, such as ARDS, ventilator-induced lung injury, and asthma, and in animal studies (Chabot et al., 1998; Lang et al., 2002; Matot et al., 2003; Nakamura et al., 1998; Wood et al., 2003). Thus, increased levels of oxidized phospholipids present in the injured lung may influence pulmonary endothelial cell (EC) functions including monocyte recruitment, modulation of pulmonary inflammatory response, and EC barrier regulation.

Intracellular signaling pathways involved in OxPAPC cellular effects are not well understood. Inhibitory effect of OxPAPC on LPS-induced E-selectin expression is attenuated by protein kinase A (PKA) inhibition, whereas OxPAPC-induced expression of early growth response factor 1 and tissue factor was abolished by inhibitors of protein kinase C (PKC) and MEK suggesting activation of PKC/MEK/Erk-1,2 pathway by OxPAPC (Bochkov et al., 2002b). A role for tyrosine kinases in oxidized LDL-mediated effects has been suggested (Maschberger et al., 2000; Yang et al., 2001); however, the specific role of OxPAPC in activation of tyrosine kinases and interaction of tyrosine kinases, MAP kinase cascade, PKC, and PKA is not clear.

An important function of pulmonary EC is regulation of mass transport in the lung. EC barrier regulation is determined by the equilibrium of competing contractile and tethering forces generated by the actomyosin cytoskeletal motor and the adhesive molecules located at cell–cell and cell–matrix contacts (Bogatcheva et al., 2002; Dudek and Garcia, 2001; Lum and Malik, 1996). We have previously demonstrated that the phosphorylation status of key cytoskeletal proteins, such as regulatory myosin light chains and cofilin, is critical for precise regulation of actomyosin-driven contraction, cytoskeletal remodeling, and EC barrier properties (Birukov et al., 2003; Garcia et al., 1995, 2001). We have also shown a critical involvement of focal adhesion proteins, paxillin, and FAK, in differential focal adhesion remodeling induced by the barrier protective phospholipid sphingosine 1-phosphate, and the barrier disruptive agonist thrombin (Schaphorst et al., 1997; Shikata et al., 2003a,b).

In this study, we characterized signaling pathways triggered by OxPAPC in human pulmonary endothelial EC and identified cytoskeletal protein targets activated by OxPAPC and involved in EC remodeling and barrier regulation.

## Materials and methods

### Materials

All biochemical reagents including mouse monoclonal pan-MLC antibody and 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (OxPAPC) were obtained from Sigma (St. Louis, MO) unless otherwise indicated. Rabbit polyclonal phospho-Raf, phospho-MEKK1/2, phospho-Erk-1,2, phospho-Elk, phospho-p90RSK, phospho-MKK4, phospho-p38, pan-p38, phospho-HSP-27, phospho-LNK, phospho-ATF-2, phospho-MLC, and phospho-paxillin antibodies, phospho-PKA substrate antibody, phospho-PKC substrate antibody, as well as MEK inhibitor UO126, were obtained from Cell Signalling (Beverly, MA). Rabbit polyclonal phospho-FAK and phospho-MYPT1 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Cell-permeable PKA peptide inhibitor, PP-2, genistein, and bisindolmaleimide I were purchased from Calbiochem (La Jolla, CA). Cell-permeable PKC peptide inhibitor was obtained from Promega Corp. (Madison, WI), rabbit polyclonal phospho-cofilin and pan-Erk1,2 antibodies were obtained from Santa Cruz (Santa Cruz, CA). Mouse monoclonal anti-FAK and anti-paxillin antibodies were obtained from BD Pharmingen (San Diego, CA).

### Cell culture

Human pulmonary artery endothelial cells were obtained from Clonetics, BioWhittaker Inc. (Frederick, MD). Cells were maintained in complete culture medium consisting of Clonetics EBM basic medium containing 10% bovine serum and supplemented with a set of nonessential amino acids, endothelial cell growth factors, and 100 units/ml penicillin/streptomycin provided by Clonetics, BioWhittaker, and incubated at 37°C in humidified 5% CO<sub>2</sub> incubator. Cells were used for experiments at passages 6–8.

### Lipid oxidation and analysis

PAPC was oxidized by exposure of dry lipid to air for 72 h. The extent of oxidation was monitored by positive ion electrospray mass spectrometry (ESI-MS) as described previously (Watson et al., 1997). Lipids were stored at –70°C in chloroform and used within 2 weeks after mass spectrometry testing. PAPC and OxPAPC preparations were shown negative for endotoxin by the limulus amoebocyte assay (BioWhittaker).

### Western immunoblotting

Protein extracts were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (30 V for 18 h or 90 V for 2 h), and the membranes were incubated with specific antibodies of interest. Equal protein loadings were verified by reprobing membranes with anti-

Erk, anti-Fax, or anti-paxillin antibodies. Immunoreactive proteins were detected with the enhanced chemiluminescent detection system (ECL) according to the manufacturer's directions (New England BioLabs, Beverly, MA). The relative intensities of the protein bands were quantified by scanning densitometry using Image Quant 5.2 (Molecular Dynamics, Piscataway, NJ) software.

#### *Activation of MAP kinase pathways and characterization of tyrosine phosphorylation*

Activation of MAP kinase cascades was monitored by Western immunoblotting techniques using phosphospecific antibodies, which are described in Materials and methods; the activated form of protein kinases of the MAP kinase cascade was also detected. Analysis of total protein tyrosine phosphorylation was performed by immunoblotting with phosphotyrosine antibody.

#### *Analysis of PKC and PKA activities*

After stimulation with OxPAPC (20 µg/ml, 15 min), HPAEC were lysed, cell lysates were clarified by centrifugation (14,000 × *g*, 5 min, +4°C), and PKA and PKC activities were measured using *in vitro* kinase assay kits obtained from Promega according to the manufacturer's protocol. Additionally, OxPAPC-induced PKC and PKA activation in HPAEC cultures was determined by immunoblotting of whole cell lysates with phospho-PKC substrate- and phospho-PKA substrate-specific antibodies that recognize PKA- or PKC-phosphorylated sites in the endogenous proteins.

#### *Statistical analysis*

ANOVAs with a Student–Newman–Keuls test were used to compare the means of two or more different treatment groups. Results are expressed as means ± SD. Differences between two groups were considered statistically significant when  $P < 0.05$ .

## **Results**

#### *OxPAPC induces activation of MAP kinase cascades*

Stimulation of HPAEC with OxPAPC (20 µg/ml) induced time-dependent activation of Erk-1,2, which peaked at 15 min and remained elevated after 30 min (Fig. 1, left panel) and 1 h (data not shown). Erk-1,2 activation by OxPAPC was associated with activation of Erk-1,2 upstream activators MEK1,2 and Raf (Fig. 1, left panel). Erk-1,2 activation resulted in phosphorylation of its downstream targets, p90RSK and Elk. Specific MEK1,2 inhibitor, UO-126 (5 µM), completely abolished OxPAPC-induced Erk-1,2, p90Rsk, and Elk phosphorylation (Fig.

1, right panel). The broad tyrosine kinase inhibitor, genistein (100 µM), and a cell-permeable peptide inhibitor of PKC (20 µM) attenuated OxPAPC-induced activation of Raf, MEK 1,2, and Erk 1,2, suggesting a role for PKC and tyrosine kinases in upstream activation of MAP kinase cascade induced by OxPAPC. Activation of MAP kinase cascades was specific for OxPAPC, as nonoxidized PAPC had no effect on Erk-1,2 activation (Fig. 1, right panel). In addition, OxPAPC preincubation with BHT, a free radical quencher, caused same levels of Erk-1,2 activation and Elk phosphorylation, as nontreated OxPAPC (Fig. 1, right panel), suggesting that effects of OxPAPC on Erk-1,2 activation are not due to residual reactive oxygen species present in OxPAPC preparation.

#### *Effects of OxPAPC on p38 and JNK MAP kinases*

In contrast to activation of the Erk-1,2 cascade, OxPAPC did not significantly increase phosphorylation of p38 and the p38-specific downstream target, HSP-27 (Fig. 2, left panel). Consistent with these observations, OxPAPC did not affect the p38 upstream activator, MKK 3/6. Analysis of JNK MAP kinase showed that OxPAPC induced phosphorylation of JNK and its downstream effector, ATF-1 (Fig. 2). OxPAPC preincubation with BHT caused same levels of JNK activation, as nontreated OxPAPC. Finally, nonoxidized PAPC was without effect on p38 and JNK MAP kinase activation (Fig. 2, right panel). Probing membranes with a pan-JNK antibody showed equal JNK content in HPAEC lysates. Stimulation of HPAEC with transforming growth factor-β (TGF-β), a known activator of p38 and JNK pathways, was used as positive control in these experiments.

#### *Activation of tyrosine phosphorylation in HPAEC by OxPAPC*

Western blot analysis of HPAEC treated with OxPAPC showed time-dependent activation of protein tyrosine phosphorylation, which peaked at 15 min and still remained elevated after 30 min of treatment (Fig. 3) and 1 h (data not shown). This activation was abolished by a broad tyrosine kinase inhibitor, genistein (100 µM) (Fig. 3, right lane). Nonoxidized PAPC was without effect on protein tyrosine phosphorylation. OxPAPC preincubation with BHT did not affect OxPAPC stimulatory effect on protein tyrosine phosphorylation (Fig. 3, right panel).

#### *OxPAPC-induced PKC activation*

Activation of PKC in HPAEC stimulated with OxPAPC was assessed using two approaches. In one series of experiments, PKC-mediated phosphorylation of endogenous protein substrates was detected by immunoblotting of HPAEC lysates with phosphospecific antibodies to PKC phosphorylation sites after OxPAPC stimulation, as described in Materials and methods. Fig. 4A depicts a profile

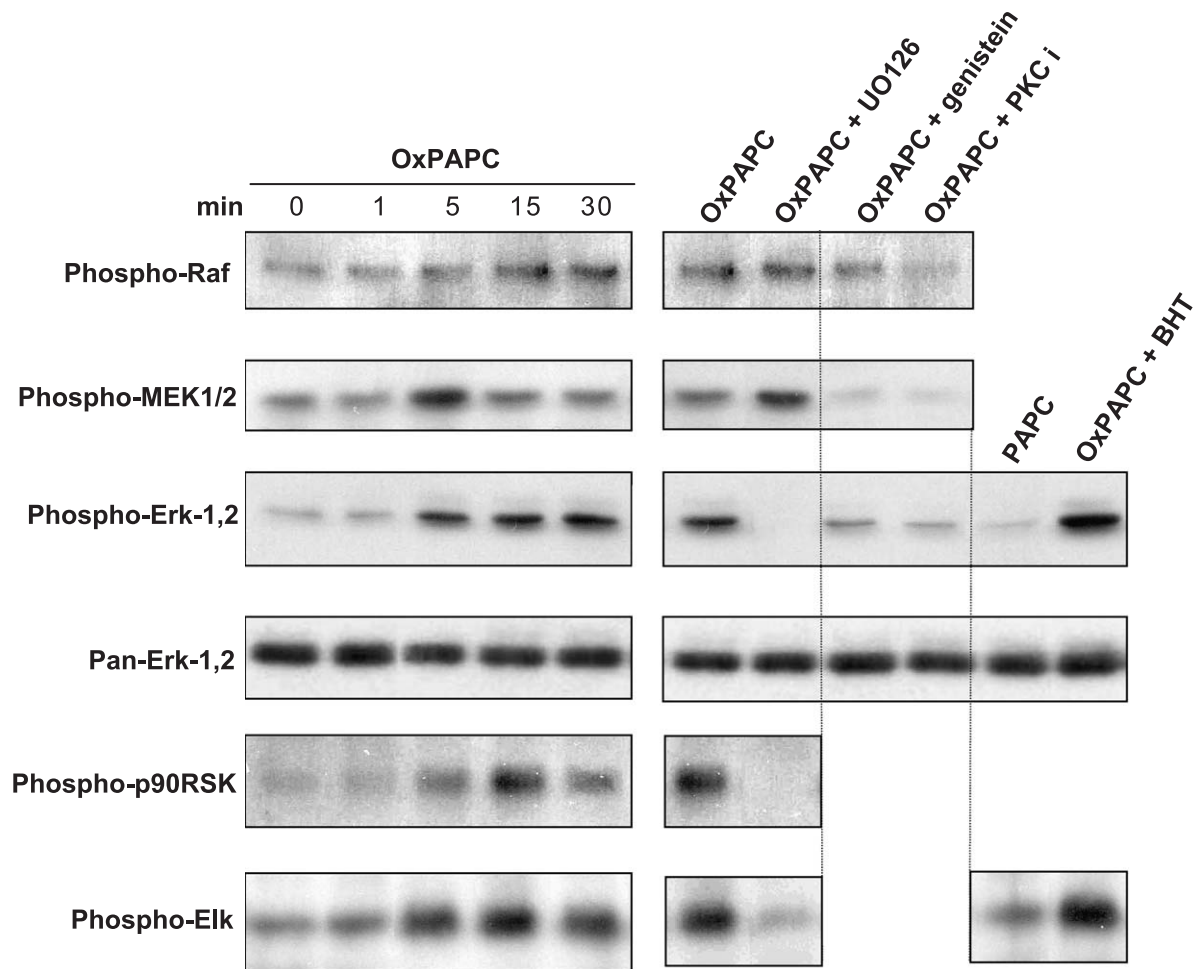


Fig. 1. Effect of OxPAPC on Raf, MEK-1,2, Erk-1,2, p90RSK, and Elk phosphorylation. HPAEC were treated with OxPAPC (20  $\mu\text{g/ml}$ ) or PAPC (20  $\mu\text{g/ml}$ ) for the indicated periods of time (left panels). On the right panels, HPAEC were pretreated for 1 h with the MEK inhibitor UO126 (5  $\mu\text{M}$ ), tyrosine kinase inhibitor genistein (100  $\mu\text{M}$ ), a cell-permeable PKC peptide inhibitor (20  $\mu\text{M}$ ), or vehicle, and stimulated with OxPAPC (20  $\mu\text{g/ml}$ , 15 min). Phosphorylation of MAP kinases and their downstream effectors was analyzed by immunoblotting of cell lysates with a panel of phosphospecific antibodies, as described in Materials and methods. Equal protein loadings were verified by membrane reprobing with a pan-Erk-1,2 antibody. Shown are representative results of three independent experiments.

of endogenous PKC-mediated protein serine/threonine phosphorylation in HPAEC and demonstrates that OxPAPC challenge induced PKC-dependent phosphorylation of a broad range of endogenous substrates with major phosphorylated proteins in the 200–240, 160, 120–130, and 70–90 kDa range. PKC activation was observed after 5 min of stimulation, peaked at 15 min, and remained elevated after 30 min of stimulation. Nonoxidized PAPC did not significantly increase endogenous protein phosphorylation (Fig. 4A, right panel). The cell-permeable specific PKC peptide inhibitor abolished OxPAPC-induced phosphorylation, thus confirming specificity of antibodies used for detection of PKC-mediated endogenous phosphorylation (Fig. 4A, right panel). Direct analysis of PKC activation in OxPAPC-stimulated HPAEC was performed in an *in vitro* kinase assay with an exogenous PKC-specific substrate peptide, as described in Materials and methods. Treatment of HPAEC with OxPAPC (20  $\mu\text{g/ml}$ , 15 min) caused a significant increase in PKC activity,

which was attenuated by the PKC peptide inhibitor (Fig. 4B). The PKC inhibitor bisindolmaleimide I attenuated OxPAPC-induced PKC activation to a lesser extent.

#### *OxPAPC-induced PKA activation*

Similar to analysis of PKC activation, assessment of PKA activity in HPAEC upon OxPAPC stimulation was performed by Western blot with antibodies specific to PKA phosphorylation sites, and in *in vitro* kinase assays. Fig. 5A depicts a profile of endogenous PKA-mediated protein serine/threonine phosphorylation in HPAEC and demonstrates that OxPAPC challenge induced PKA-dependent phosphorylation of a broad range of endogenous substrates with major phosphorylated proteins in the 200–220, 140–160, 130, and 80–90 kDa range. PKA activation was observed after 5 min of stimulation, peaked at 15 min, and remained elevated after 30 min of stimulation. Cell-permeable specific PKA peptide inhibitor abolished

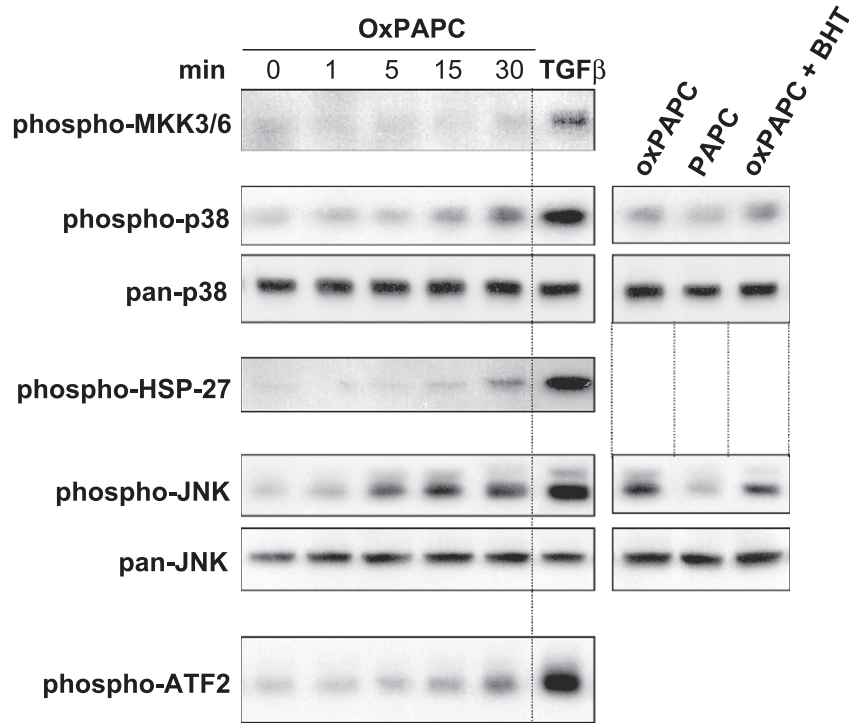


Fig. 2. Effect of OxPAPC on MKK 3/6, p38, HSP-27, JNK, and ATF-1 phosphorylation. Left panel: time course of OxPAPC-mediated activation of p38 and JNK MAP kinase cascade. HPAEC were treated with OxPAPC (20 μg/ml) for the indicated periods of time. TGF-β (10 ng/ml, 30 min) was used as positive control for p38 and JNK activation. Right panels: HPAEC were incubated with OxPAPC (20 μg/ml), PAPC (20 μg/ml), or OxPAPC preincubated for 10 min with the free radical blocker BHT (10 μM). Phosphorylation of MAP kinases and their downstream effectors was analyzed by immunoblotting with a panel of phosphospecific antibodies, as described in Materials and methods. Equal protein loadings were verified by membrane reprobing with pan-p38 and pan-JNK antibodies. Shown are representative results of three independent experiments.

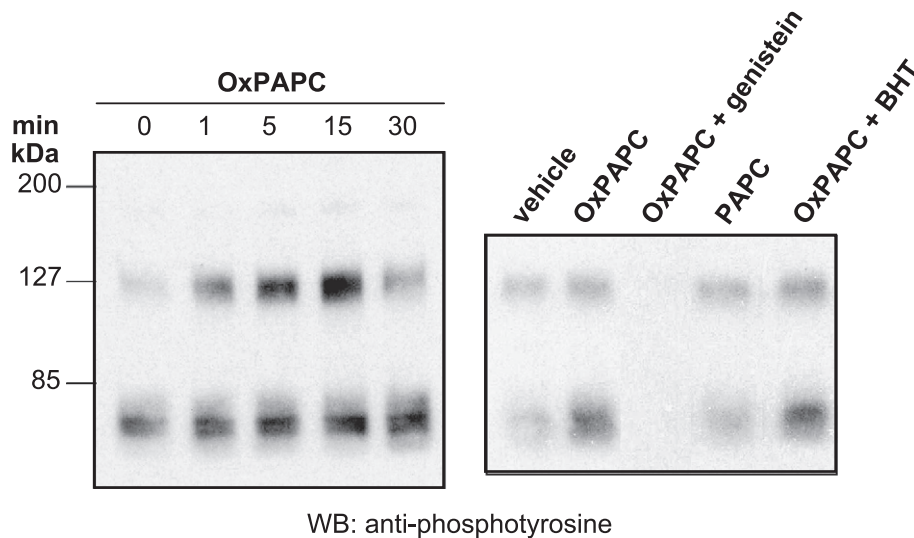


Fig. 3. OxPAPC increases protein tyrosine phosphorylation. Left panel: time course of OxPAPC-induced protein tyrosine phosphorylation. HPAEC were treated with OxPAPC (20 μg/ml) for the indicated periods of time. Right panel: HPAEC were pretreated for 1 h with tyrosine kinase inhibitor genistein (100 μM) or vehicle, and stimulated for 15 min with OxPAPC (20 μg/ml), PAPC (20 μg/ml), or OxPAPC preincubated for 10 min with BHT (10 μM). Total protein tyrosine phosphorylation was detected on immunoblot with antiphosphotyrosine antibody, as described in Materials and methods. Equal protein loadings were verified by membrane reprobing with pan-Erk-1,2 antibodies (data not shown). OxPAPC induces time-dependent activation of protein tyrosine phosphorylation, which was abolished by genistein and was not affected by OxPAPC pretreatment with BHT. PAPC does not increase protein tyrosine phosphorylation. Shown are representative results of three independent experiments.

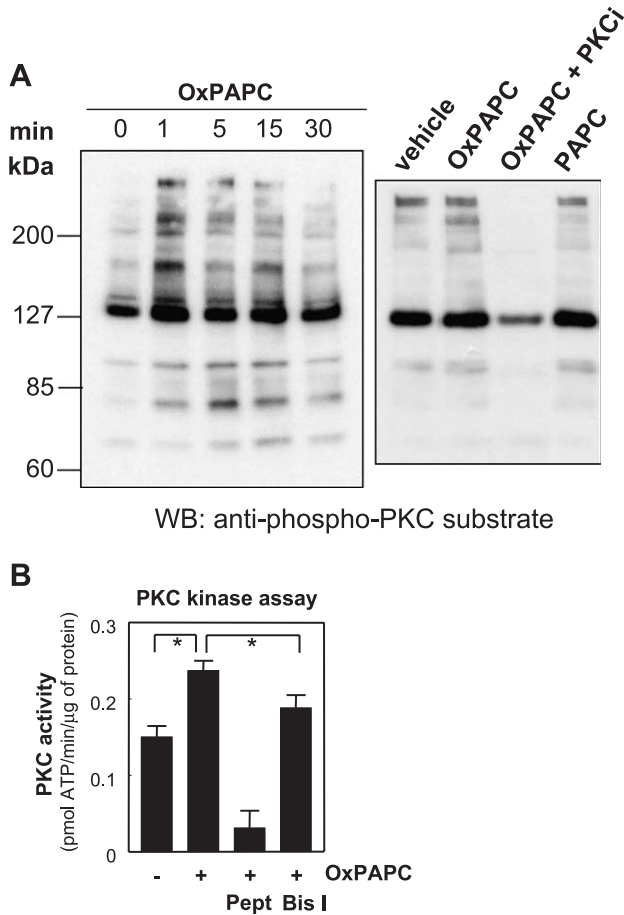


Fig. 4. OxPAPC-induced activation of protein kinase C. (A) HPAEC were treated with OxPAPC (20  $\mu\text{g}/\text{ml}$ ) for the indicated periods of time, and PKC-mediated phosphorylation of endogenous substrates was monitored by immunoblotting with anti-phospho-PKC substrate antibody, as described in Materials and methods. Right panel: HPAEC were pretreated with a cell-permeable PKC peptide inhibitor (20  $\mu\text{M}$ ) 1 h before OxPAPC stimulation, or cells were treated with OxPAPC or PAPC (20  $\mu\text{M}$ ) alone. Equal protein loadings were verified by membrane reprobing with pan-Erk-1,2 antibodies (data not shown). Shown are representative results of three independent experiments. (B) HPAEC stimulated with OxPAPC (20  $\mu\text{g}/\text{ml}$ , 15 min) were lysed, and PKC activity in cell lysates was determined in an in vitro kinase assay, as described in Materials and methods. HPAEC preincubation with PKC peptide inhibitor and bisindolmaleimide I (1  $\mu\text{M}$ ) was performed for 1 h before OxPAPC stimulation. PKC activity is expressed as picomoles of phosphate incorporated per milligram of protein per minute. Results are mean  $\pm$  SD of three independent experiments. \* $P < 0.05$ .

OxPAPC-induced phosphorylation, thus confirming specificity of antibodies used for detection of PKA-mediated endogenous phosphorylation (Fig. 4A, right panel). In vitro PKA kinase assay showed that OxPAPC also increased PKA activity, which was attenuated by a cell-permeable PKA peptide inhibitor (Fig. 5B). Nonoxidized PAPC did not induce PKA activation (Fig. 5A, right panel).

#### Effects of OxPAPC on cytoskeletal proteins

OxPAPC-mediated activation of PKC and tyrosine phosphorylation may induce changes in cytoskeletal organization

and cell contact arrangement. In the next series of experiments, we examined effects of OxPAPC on potential cytoskeletal and cell adhesion protein targets.

Phosphorylation of regulatory myosin light chains triggers actin stress fiber assembly, cytoskeletal rearrangement, actomyosin contraction, and may lead to endothelial cell retraction and gap formation (for a review, see Dudek and Garcia, 2001). Along with MLC kinases, myosin-specific phosphatase (MYPT1) plays a critical role in regulation of MLC phosphorylation status. Phosphorylation of Thr<sup>686</sup> and Thr<sup>850</sup> leads to MYPT1 inactivation and thus increases MLC phosphorylation (Carbajal et al., 2000; Velasco et al., 2002). OxPAPC treatment did not affect MLC phos-

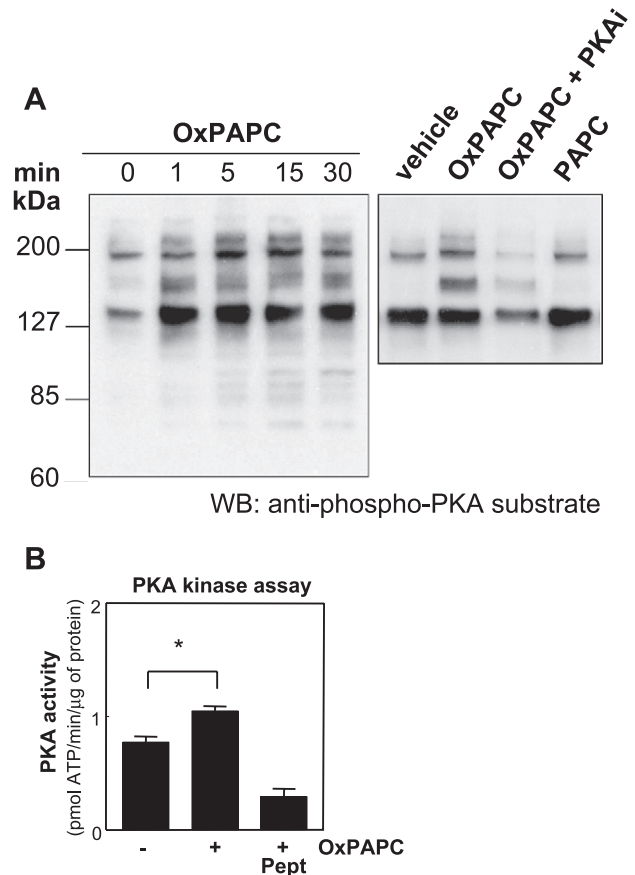


Fig. 5. OxPAPC-induced protein kinase A activation. (A) HPAEC were treated with OxPAPC (20  $\mu\text{g}/\text{ml}$ ) for the indicated periods of time, and PKA-mediated phosphorylation of endogenous substrates was monitored by immunoblotting with anti-phospho-PKA substrate antibody, as described in Materials and methods. Right panel: HPAEC were pretreated with cell-permeable PKA peptide inhibitor (20  $\mu\text{M}$ ) 1 h prior to OxPAPC stimulation, or cells were treated with OxPAPC or PAPC (20  $\mu\text{M}$ ) alone. Equal protein loadings were verified by membrane reprobing with pan-Erk-1,2 antibodies (data not shown). Results are representative of three independent experiments. (B) HPAEC stimulated with OxPAPC (20  $\mu\text{g}/\text{ml}$ , 15 min) were lysed, and PKA activity in cell lysates was determined in in vitro kinase assay, as described in Materials and methods. HPAEC preincubation with PKA peptide inhibitor (20  $\mu\text{M}$ ) was performed for 1 h prior to OxPAPC stimulation. PKA activity is expressed as picomoles of phosphate incorporated per milligram of protein per minute. Results are mean  $\pm$  SD of three independent experiments. \* $P < 0.05$ .

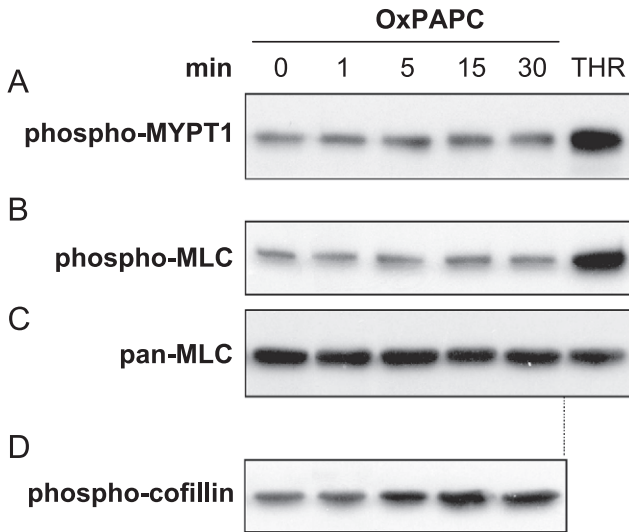


Fig. 6. Effect of OxPAPC on phosphorylation of MYPT-1, MLC, and cofillin. HPAEC were treated with OxPAPC (20 µg/ml) for the indicated periods of time, and phosphorylation of MYPT-1, MLC, and cofillin was detected by immunoblotting with corresponding phosphospecific antibody, as described in Materials and methods. Equal protein loadings were verified by membrane reprobing with a pan-MLC antibody. Shown results are representative of three independent experiments.

phorylation levels, as detected by Western blot with anti-diphospho-MLC antibody raised against a MLC epitope containing phospho-Ser<sup>19</sup> and phospho-Thr<sup>18</sup> (Fig. 6B). Panel C depicts equal MLC content in the samples.

OxPAPC also did not affect MYPT site-specific phosphorylation, as examined by immunoblotting HPAEC lysates with a blend of MYPT anti-Thr<sup>686</sup> and anti-Thr<sup>850</sup> antibodies (Fig. 6A). However, OxPAPC treatment induced significant phosphorylation of cofillin, an actin-binding protein involved in regulation of actin polymerization (Fig. 6D).

*Effects of OxPAPC on FAK and paxillin phosphorylation*

FAK and paxillin are focal adhesion proteins involved in cell motility and focal adhesion remodeling (Parsons et al., 2000; Turner, 2000). OxPAPC treatment induced time-dependent tyrosine phosphorylation of FAK at Tyr<sup>576</sup>, a site critical for activation of FAK catalytic activity (Parsons et al., 2000), and paxillin at Tyr<sup>118</sup>, the site of phosphorylation by FAK (Turner, 2000; Fig. 7). Equal FAK and paxillin loadings were verified with pan-FAK and pan-paxillin antibodies. OxPAPC-induced phosphorylation of FAK and paxillin was attenuated by HPAEC pretreatment with p60Src-specific inhibitor PP-2 (5 µM) before OxPAPC stimulation (Fig. 7, right panel).

**Discussion**

Oxidized LDL induce diverse physiological responses in vascular smooth muscle and endothelial cells, which include activation of cell proliferation, expression of inflammatory

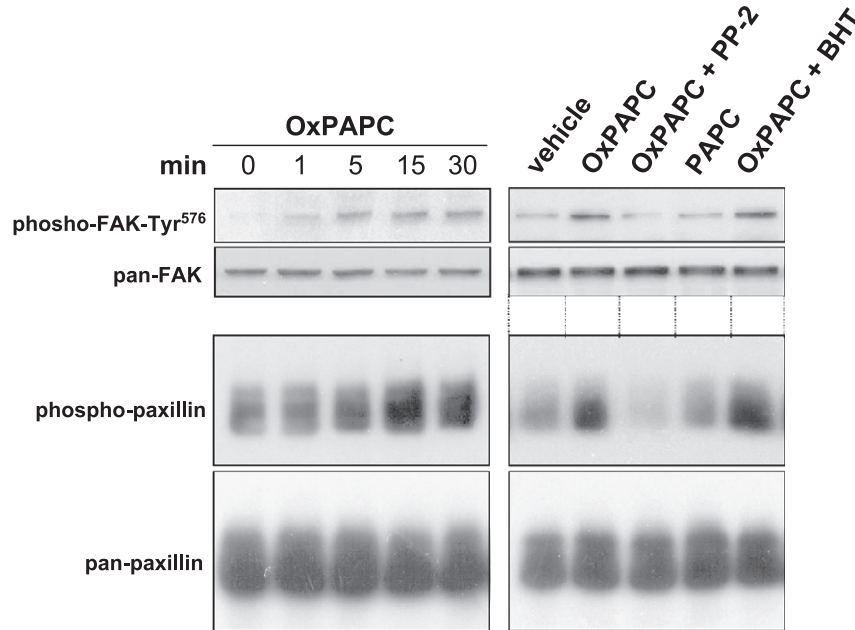


Fig. 7. Effect of OxPAPC on phosphorylation of paxillin and FAK. Left panel: HPAEC were treated with OxPAPC (20 µg/ml) for the indicated periods of time. Right panel: HPAEC were pretreated with p60Src-specific inhibitor PP-2 (1 µM) or vehicle for 1 h and stimulated with OxPAPC (20 µg/ml, 15 min), or treated with PAPC (20 µg/ml), or with OxPAPC preincubated for 10 min with BHT (10 µM). Phosphorylation of paxillin-Tyr<sup>118</sup> and FAK-Tyr<sup>576</sup> was detected by immunoblotting with the corresponding phosphospecific antibody, as described in Materials and methods. Equal protein loadings were verified by membrane reprobing with pan-paxillin and pan-FAK antibodies. Shown results are representative of three independent experiments.

adhesion molecules, activation of actomyosin contraction, or activation of apoptosis (Essler et al., 1999; Leitinger et al., 1999; Li et al., 1998; Mine et al., 2002; Napoli et al., 2000; Yang et al., 2001). Apparent inconsistency of cellular responses induced by oxidized LDL may be due to heterogeneity of LDL components (Leitinger et al., 1999; Watson et al., 1997), different LDL oxidation conditions used by investigators, and by cell-type specificity of responses (Li et al., 1998; Yang et al., 2001).

OxPAPC is a bioactive component of OxLDL and oxidized cell membranes with well-characterized chemical properties (Watson et al., 1997). OxPAPC induces monocyte adhesion to vascular endothelium from systemic circulation and exhibits antagonistic effects on expression of pro-inflammatory surface receptors (VCAM and E-selectin) and adhesion of neutrophils to endothelial cells induced by LPS (Bochkov et al., 2002a; Leitinger et al., 1999). Inhibitory analysis of signaling pathways triggered by OxPAPC linked physiological effects of OxPAPC to several signaling molecules such as protein kinase A (Leitinger et al., 1999), protein kinase C, and Erk-1,2 (Bochkov et al., 2002b). However, precise mechanisms of OxPAPC-mediated intracellular signaling have not been yet investigated. In this study, we characterized effects of OxPAPC on intracellular signaling in human pulmonary endothelial cells. Our results suggest a rapid activation of PKC, PKA, protein tyrosine phosphorylation, and MAP kinase cascades by OxPAPC. Moreover, inhibition of PKC and tyrosine kinase activities attenuated activation of Raf, MEK-1,2, and Erk-1,2. One potential PKC-dependent mechanism involves PKC-mediated inactivation of Ras GTPase activating protein (Ras GAP), which is a negative regulator of GTPase Ras, which in turn activates Raf (Gutkind, 1998). Tyrosine phosphorylation may play a role in OxPAPC-induced activation of Raf via p60Src-mediated mechanisms (Luttrell et al., 1999; Porter and Vaillancourt, 1998). OxPAPC did not activate the p38 MAP kinase cascade, but modestly activated JNK and induced phosphorylation of ATF-2. Although ATF-1 is a substrate for both p38 and JNK MAP kinases, its phosphorylation upon OxPAPC treatment is most likely attributed to JNK activation. Differential activation of MAP kinase cascades is consistent with previous findings suggesting Erk-1,2-dependent mechanisms for activation of Egr and tissue factor expression observed in endothelial cells from systemic circulation (Bochkov et al., 2002b). Results of this study demonstrate OxPAPC-mediated activation of Erk-1,2 substrates, p90RSK, and Elk involved in transcriptional regulation, and suggest a potential role for the JNK effector ATF-2 in OxPAPC-induced specific gene expression in human pulmonary EC.

Activation of PKA and PKC in OxPAPC-stimulated pulmonary EC may dually impact cell function. Increased intracellular cAMP levels and consequent activation of cAMP-dependent protein kinase (PKA) exhibit protective effects on vascular leak induced by inflammatory mediators, such as thrombin, phorbol myristoyl acetate (PMA),

pertussis toxin, and bacterial wall lipopolysaccharide (LPS) (Adkins et al., 1993; Chetham et al., 1997; Essler et al., 2000; Garcia et al., 1995; Liu et al., 2001; Patterson et al., 1994, 2000). Molecular mechanisms of barrier protective effects of PKA include: (1) PKA-mediated phosphorylation of endothelial myosin light chain kinase (MLCK) and attenuation of its activity leading to decreased basal level MLC phosphorylation (Garcia et al., 1995, 1997); (2) phosphorylation of actin-binding proteins, filamin, adductin, and dematin (Hastie et al., 1997; Matsuoka et al., 1996; Wallach et al., 1978), and focal adhesion proteins, paxillin and FAK, which leads to disappearance of stress fibers and F-actin accumulation in the membrane ruffles (Han and Rubin, 1996; Troyer et al., 1996); (3) PKA-mediated modulation of Rho GTPase activity. PKA can phosphorylate RhoA at Ser<sup>188</sup> (Lang et al., 1996) and thus decrease Rho association with Rho kinase (Busca et al., 1998; Dong et al., 1998). PKA activation also increases the interaction of RhoA with Rho-GDP dissociation inhibitor (Rho-GDI) and translocation of RhoA from the membrane to the cytosol (Lang et al., 1996; Qiao et al., 2003; Tamma et al., 2003). Thus, the overall effect of PKA on RhoA is downregulation of RhoA activity and stabilization of cortical actin cytoskeleton, which may promote EC barrier properties. Activation of PKC by phorbol esters induces specific cytoskeletal remodeling and exhibits barrier-disruptive effects on macrovascular EC; however, it promotes barrier-protective responses in lung microvascular EC (Bogatcheva et al., 2003). In addition, recent studies demonstrate that monolayer permeability changes are differentially regulated by PKC isoenzymes, suggesting that PKC alpha promotes endothelial barrier dysfunction and PKC delta enhances basal endothelial barrier function (Harrington et al., 2003). Further studies aimed at analysis of isoform-specific PKC activation will shed a light on the role of PKC isoforms in OxPAPC-induced cell signaling and endothelial cell function.

Although kinetics of OxPAPC-mediated intracellular signaling suggests a receptor type of cellular response, the specific receptor for OxPAPC has not yet been identified. Some, but not all, effects of OxPAPC, can be partially attenuated by platelet activating factor (PAF) receptor antagonists (Kadl et al., 2002; Leitinger et al., 1997), whereas PAF itself does not mimic OxPAPC effects (Leitinger et al., 1997). These observations suggest potential structural homology of a putative OxPAPC receptor with the PAF receptor.

In this study, we also examined potential downstream cytoskeletal targets of OxPAPC-mediated signaling. Previous reports suggest that oxidized LDL may cause Rho-mediated stress fiber formation, robust MLC phosphorylation in endothelial cells, and actin polymerization in platelets (Essler et al., 1999; Maschberger et al., 2000). Results of our study suggest that OxPAPC did not increase the levels of MLC phosphorylation in HPAEC. Moreover, site-specific analysis of MYPT1 phosphorylation sites, Thr<sup>686</sup> and Thr<sup>850</sup>, which are specific sites for phosphory-

lation by Rho-associated kinase (Carbajal et al., 2000; Velasco et al., 2002), showed no changes in phosphorylation after OxPAPC treatment. These results clearly indicate that OxPAPC treatment does not increase MLC phosphorylation, which is tightly linked to actomyosin contraction in HPAEC (Dudek and Garcia, 2001). However, we observed increases in phosphorylation of cofilin, an actin binding protein involved in regulation of actin polymerization. Nonphosphorylated cofilin binds actin monomers and prevents actin polymerization, whereas cofilin phosphorylation abolishes cofilin–actin interaction and thus promotes actin polymerization (Chen et al., 2000; Cooper and Schafer, 2000). Thus, our results strongly suggest involvement of OxPAPC in HPAEC actin remodeling via cofilin phosphorylation, and further studies are under way to more precisely characterize human pulmonary EC remodeling induced by OxPAPC. Consistent with proposed cytoskeletal effects of OxPAPC, we demonstrate that OxPAPC challenge also induced phosphorylation of focal adhesion proteins paxillin and focal adhesion kinase (FAK). Paxillin is a multidomain adapter focal adhesion protein containing binding sites for various signaling molecules and structural proteins (Birge et al., 1993; Turner and Miller, 1994; Turner et al., 1990). Paxillin facilitates signal transduction from extracellular matrix and receptor-dependent agonists by recruiting specific molecules to focal adhesions, and paxillin phosphorylation by FAK at Tyr<sup>118</sup> is important for determining its binding partners (Bellis et al., 1995; Schaller and Parsons, 1995; Turner, 1998). In turn, FAK autophosphorylation and phosphorylation by other tyrosine kinases, such as p60Src, is a major mechanism for regulation of FAK catalytic activity and interaction with binding partners (Parsons et al., 2000; Schaller, 2001). Therefore, increased FAK and paxillin tyrosine phosphorylation in OxPAPC-stimulated HPAEC and its attenuation by specific P60Src inhibitor, PP-2, suggest effects of OxPAPC on focal adhesion remodeling, which may be mediated by p60Src and FAK.

In summary, this study provides for the first time a comprehensive analysis of OxPAPC-mediated signaling and suggests potential effects of oxidized phospholipids on specific gene expression and cytoskeletal remodeling in EC from pulmonary circulation. We described OxPAPC-mediated activation of MAP kinase cascades and PKC and PKA catalytic activities in human pulmonary endothelium. We demonstrated activation of specific regulatory proteins, cofilin, paxillin, and FAK, involved in remodeling of actin cytoskeleton and cell focal adhesions. Taken together with stimulatory effects of OxPAPC on tissue factor expression and monocyte adhesion to endothelium, previously described in systemic circulation (Bochkov et al., 2002b; Leitinger et al., 1997; Subbanagounder et al., 2000), our data suggest a novel role for oxidized phospholipids in pulmonary circulation related to modulation of lung inflammatory response and EC cytoskeletal changes.

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