Atherosclerosis and Lipoproteins

Vascular Endothelial Growth Factor Receptor 2 Plays a Role in the Activation of Aortic Endothelial Cells by Oxidized Phospholipids

Alejandro Zimman, Kevin P. Mouillesseaux, Thang Le, Nima M. Gharavi, Ann Ryvkin, Thomas G. Graebner, Tom T. Chen, Andrew D. Watson, Judith A. Berliner

Objective—Previous studies have shown that oxidized products of PAPC (Ox-PAPC) regulate cell transcription of interleukin-8, LDL receptor, and tissue factor. This upregulation takes place in part through the activation of sterol regulatory element-binding protein (SREBP) and Erk 1/2. The present studies identify vascular endothelial growth factor receptor 2 (VEGFR2) as a major regulator in the activation of SREBP and Erk 1/2 in endothelial cells activated by Ox-PAPC.

Methods and Results—Ox-PAPC induced the phosphorylation of VEGFR2 at Tyr1175 in human aortic endothelial cells. Inhibitors and siRNA for VEGFR2 decreased the transcription of interleukin-8, LDL receptor, and tissue factor in response to Ox-PAPC and the activation of SREBP and Erk 1/2, which mediate this transcription. We provide evidence that the activation of VEGFR2 is rapid, sustained, and c-Src–dependent.

Conclusions—These data point to a major role of VEGFR2 in endothelial regulation by oxidized phospholipids which accumulate in atherosclerotic lesions and apoptotic cells. 

Key Words: oxidized phospholipids • VEGFR2 • atherosclerosis • endothelium

Monocyte adhesion to the endothelium and migration into the subendothelial space are early events in the chronic inflammatory process of atherosclerosis.1 Oxidized products of 1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphorylcholine (Ox-PAPC), a component of minimally modified low-density lipoprotein (mm-LDL), activate endothelial cells and increase monocyte-endothelial interactions in vitro.2 Active lipid components of Ox-PAPC accumulate in atherosclerotic lesions3 as well as in apoptotic cells.4 Recently, increased levels of oxidized phospholipids in blood have been correlated with the risk of coronary artery disease,5 adding more evidence to the hypothesis that Ox-PAPC has an important role in the early stages of atherosclerosis.

Treatment of endothelial cells in culture with Ox-PAPC results in the transcriptional activation of a large number of genes.6 The response includes the activation of inflammatory, sterol sensing, redox, unfolded protein response, cell cycle, and coagulation genes. For example, Ox-PAPC increases the transcription of LDL receptor (LDL-R) and the secretion of chemokines such as interleukin-8 (IL-8) and monocyte chemotactic protein-1.7 Interestingly, the activation of IL-8 promoted by Ox-PAPC is more prolonged (over 24 hours) than the activation by tumor necrosis factor (TNF)-α (less than 6 hours) and independent of NF-κB.8 Our group has reported two independent signal transduction pathways responsible for the induction of IL-8 by Ox-PAPC: the c-Src/signal transducers and activators of transcription (STAT)-3 pathway9 and the activation of the sterol regulatory element-binding protein (SREBP)-1c.10 Furthermore, Ox-PAPC was shown to induce tissue factor (TF) transcription via activation of Erk 1/2.11 These studies identify at least 3 independent signal transduction pathways activated by Ox-PAPC.

The fact that Ox-PAPC induces so many genes and that independent pathways are activated suggests a large network of signaling. In contrast to the progress in the identification of signal transduction pathways activated by Ox-PAPC in endothelial cells, only one receptor responsive to Ox-PAPC has been clearly identified, prostaglandin (PG) E2 receptor subtype 2.12 While this receptor appears to mediate integrin activation in endothelial cells, no clear role for this receptor in regulation of transcription has been identified.

In the present studies, we report that VEGFR2 plays a role in the upregulation by Ox-PAPC of mRNA levels for inflammatory, sterol sensing, and coagulation genes.

Materials and Methods

Materials
Human VEGF 165 was obtained from R&D Systems (Minneapolis, MN). Inhibitor for VEGFR2 (SU-1498) and c-Src (PP2) were...
purchased from Calbiochem (San Diego, Calif). Rabbit anti-human VEGFR2, phospho-VEGFR2 (Tyr1175), p44/42 MAPK (Erk1/2), and phospho-p44/42 MAPK (Thr202/Tyr204) were purchased from Cell Signaling (Beverly, Mass). Rabbit anti-SREBP-1 (K-10 and H-160) were from Santa Cruz Biotechnology (Santa Cruz, Calif). Rabbit anti-human VEGF was obtained from Chemicon (Temecula, Calif). M199 medium and DME high glucose were from Irvine Scientific (Santa Ana, Calif) and fetal bovine serum (FBS) was purchased from HyClone (Logan, Utah). HiPerFect transfection reagent was from Qiagen (Valencia, Calif). PAPC was purchased from Avanti Polar Lipids (Alabaster, Ala) and oxidized to Ox-PAPC as previously described. PEIPC was prepared as described before. Lipofectamine 2000 and OptiMEM I reduced serum medium were obtained from Invitrogen (Carlsbad, Calif). HiPerFect transfection reagent was from Qiagen (Valencia, Calif). M199 medium and DME high glucose were from Irvine Scientific (Santa Ana, Calif) and fetal bovine serum (FBS) was purchased from HyClone (Logan, Utah).

**Cell Culture and Treatments**

Human aortic endothelial cells (HAECs) were isolated as described previously and cultured in M199 medium supplemented with FBS (20% v/v), penicillin (100 U/mL), streptomycin (100 µg/mL), sodium pyruvate (1 mmol/L), heparin (90 µg/mL), and endothelial cell growth supplement (20 µg/mL). For treatments, Ox-PAPC was dried to a lipid residue, resuspended in media supplemented with 1% v/v FBS, and added to the cells. For chemical inhibitor studies, HAECs were pretreated with the inhibitor dissolved in DMSO or an equivalent volume of DMSO for 1 hour. Afterward, the cells were cotreated with Ox-PAPC and the inhibitor (or DMSO) for the times indicated.

HEK 293 cells stably expressing VEGFR2 were provided by Dr Bruce I. Terman (Albert Einstein College of Medicine, New York) and grown in DME high glucose with FBS (10% v/v). Cell treatment was conducted under the same conditions described for HAECs.

**Enzyme-Linked Immunosorbent Assay**

Levels of IL-8 in supernatants of HAECs were measured for the decrease of VEGFR2 mRNA measured by quantitative real-time polymerase chain reaction (qRT-PCR). The siRNA selected for transfection into HAECs had the sequences: 5’AAUACUCUGUCGCUAUCUGUGA-3’ and 5’CCUGGAGAACACAGCGCACAUGG-3’. Values were normalized to GAPDH with the sequences: 5’CCCTCAAGATCATAGCAATGCCTCCTCA-3’ and 5’GGTGCATGCTTCCTCCAGTACACCA-3’.

**Immunoblotting**

HAECs and HEK 293 cells were lysed with 50 mmol/L Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 2 mmol/L Na3VO4, and 1 mmol/L NaF. Protein concentration was determined using the BCA assay (Pierce). Equal amounts of proteins from each sample were loaded into the gel for electrophoresis. Proteins were then transferred to polyvinylidene fluoride membranes and probed with specified antibodies. Chemiluminescence levels were detected using a Bio-Rad Versadoc Imaging System (Model 5000) and band intensity was measured by densitometry. In HAECs the antibody against VEGFR2 recognizes two bands but only the upper band is recognized by an antibody raised against phospho-tyrosine VEGFR2 (Tyr1175).

**qRT-PCR**

RNA was isolated from HAECs using the RNeasy kit from Qiagen following the manufacturer’s instructions. cDNA was obtained using iScript cDNA synthesis kit from BioRad. qRT-PCR measurements were conducted in triplicate using an iCycler IQ from BioRad. Primers to measure relative changes in IL-8 mRNA had the sequences: 5’ ACCACACTGCGCAACAGAAT3’ and 5’TCCAGACAGAGTCTTCTTCCATCAGA3’ for LDL receptor mRNA the sequences were: 5’CGTCCTTGTCTGCTACATT3’ and 5’AGAATCAGAAGCAGCGGTGTA3’. For tissue factor the mRNA sequences were: 5’TTTGGAGTGGAACCTACTAT3’ and 5’ACCGTGCCAAGTACGTGCTTCACAT3’. Values were normalized to GAPDH with the sequences: 5’CCCTCAAGATCATAGCAATGCCTCCTCA-3’ and 5’GGTGCATGCTTCCTCCAGTACACCA-3’.

**Figure 1.** Ox-PAPC induces the phosphorylation of VEGFR2 at Tyr1175. HAECs were incubated with media alone (C) or 30 µg/mL Ox-PAPC (Ox) for (a) 5, 15, 30, and 60 minutes and (b) 5 minutes, 2 hours and 4 hours. For a and b equal amounts of protein were separated by gel electrophoresis and probed with an antibody raised against phospho-tyrosine VEGFR2 (Tyr1175) (top). The blot was then reprobed with VEGFR2 antibody for normalization (middle). The fold increase in phosphorylation induced by Ox-PAPC is calculated by comparison to the phosphorylation in cells treated with media alone for the same amount of time (bottom). Similar results were obtained from 3 independent experiments.
Results

Ox-PAPC Promotes the Phosphorylation of VEGFR2 at Tyr1175

The phosphorylation of VEGFR2 at Tyr1175 has a central role in the activation of this receptor.14 Immunoblotting showed that the phosphorylation of Tyr1175 occurred within 5 minutes and increased for at least 4 hours after Ox-PAPC treatment (Figure 1a and 1b). Phosphorylation of Tyr1175 by Ox-PAPC was observed at a concentration as low as 6 μg/mL. Higher concentrations of up to 50 μg/mL induced greater phosphorylation at this site after 5 minutes of incubation with Ox-PAPC (please see supplemental data, available online at http://atvb.ahajournals.org). The phosphorylation of Tyr1175 by Ox-PAPC was completely inhibited by SU-1498 (unpublished data, 2006), a chemical inhibitor that prevents the phosphorylation of VEGFR2.15

Inhibition of VEGFR2 Activity Reduces Ox-PAPC Mediated Expression of IL-8 and LDL-R

The change in phosphorylation of VEGFR2 by Ox-PAPC led us to determine whether inhibition of this receptor affects any of the known responses to Ox-PAPC in endothelial cells. Pretreatment of HAECs with SU-1498 significantly lowered the induction of IL-8 and LDL-R mRNA by Ox-PAPC (Figure 2a). Because Ox-PAPC is a mixture of several oxidized phospholipids, we examined the effect of SU-1498 on the most bioactive component of Ox-PAPC, 1-palmitoyl-2-epoxyisoprostane E2, sn-glycerol-3-phosphorylcholine (PEIPC). SU-1498 also decreased the mRNA levels of IL-8 and LDL-R mRNA induced by 500 ng/mL of PEIPC (Figure 2b).

We used an RNAi approach to verify the role of VEGFR2 in Ox-PAPC signaling. Using siRNA, we reduced the expression of VEGFR2 in endothelial cells as much as 83% measured by immunoblotting without any significant change in VEGFR1 (please see supplemental data). Relative levels of VEGFR3 mRNA were very low compared with VEGFR1 and VEGFR2 but they also show no change. siRNA targeting VEGFR2 significantly reduced the levels of IL-8 mRNA (Figure 3a) and LDL-R mRNA (Figure 3b) induced by Ox-PAPC. The same genes were also induced by VEGF in HAECs in agreement with previous reports16,17 and siRNA against VEGFR2 inhibits their upregulation by the ligand (Figure 3a and 3b). These findings demonstrate the role of VEGFR2 in Ox-PAPC regulated transcription in HAECs.

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** A chemical inhibitor of VEGFR2 reduces the synthesis of IL-8 and LDL-R induced by Ox-PAPC and PEIPC. a, HAECs were incubated for 1 hour with or without 1 μmol/L SU-1498; then 40 μg/mL Ox-PAPC or media alone was added to the cells for 4 hours. b, HAECs were incubated for 1 hour with or without 10 μmol/L SU-1498; then 500 ng/mL PEIPC or media alone was added to the cells for 4 hours. For a and b relative change in mRNA IL-8 and LDL-R were measured by qRT-PCR and normalized to GAPDH. Data represent mean±SD (n=3). **P<0.01 and *P<0.05 compared with cells treated without inhibitor using student t test.

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** A siRNA to VEGFR2 reduces the synthesis of IL-8 and LDL-R induced by Ox-PAPC and VEGF. HAECs were transfected with 5 nmol/L of siRNA VEGFR2 (siRNA VEGFR2) or 5 nmol/L of scrambled siRNA (SCR). a, After transfection cells were incubated with different concentrations of Ox-PAPC, 50 ng/mL VEGF, or media alone for 2 hours. Relative change in mRNA IL-8 was measured by qRT-PCR and normalized to GAPDH. b, After transfection cells were incubated with 30 μg/mL Ox-PAPC, 50 ng/mL VEGF, or media alone for 2 hours. Relative change in mRNA LDL-R was measured by qRT-PCR and normalized to GAPDH. For a and b data represent mean±SD (n=3). **P<0.01 and *P<0.05 compared with cells transfected with scrambled siRNA using student t test.
Inhibition of VEGFR2 Reduces the Activation of SREBP-1 by Ox-PAPC

Our previous studies suggested that activation of SREBP-1 regulates the sustained transcription of IL-8 and LDL-R. Activation of SREBP-1 is measured by a decrease in the precursor form (125 kDa) and an increase in the mature form (68 kDa) of SREBP. Pretreatment of HAECs with the inhibitor SU-1498 decreased the ratio of mature to precursor form from 8 to 2 in cells treated with Ox-PAPC (Figure 4a). These studies show that VEGFR2 is involved in the SREBP-1 activation by Ox-PAPC that results in the upregulation of IL-8 and LDL-R.

Inhibition of VEGFR2 Also Inhibits the Effect of Ox-PAPC on Tissue Factor Synthesis and the Phosphorylation of Erk 1/2

We also examined the role of VEGFR2 in another signal transduction pathway activated by Ox-PAPC, Erk 1/2 phosphorylation, which regulates the transcription of TF by Ox-PAPC. Ox-PAPC caused an increase in Erk 1/2 phosphorylation in HAECs transfected with a scrambled siRNA, whereas specific siRNA to VEGFR2 reduced the phosphorylation levels (Figure 4b). Incubation of these cells with Ox-PAPC for longer periods of time up to 4 hours also did not show any change in phosphorylation (unpublished data, 2006). In addition, a reporter gene assay using an IL-8 promoter construct was transfected into HEK 293 cells expressing VEGFR2 and treated with VEGF or Ox-PAPC. VEGF was able to induce reporter activation in these cells whereas Ox-PAPC did not

Possible Mechanisms of VEGFR2 Activation by Ox-PAPC

We next examined whether the activation of VEGFR2 induced by Ox-PAPC was mediated by an autocrine stimulation resulting from the rapid release of VEGF by endothelial cells. Pretreatment of HAECs with different concentrations of VEGF neutralizing antibody had no effect on the level of IL-8 secreted into the media in response to Ox-PAPC. VEGF neutralizing antibody, however, was effective at reducing the level of IL-8 secreted by cells treated with 10 and 50 ng/mL of VEGF (Figure 5). These studies demonstrate that Ox-PAPC does not produce a sufficient release of VEGF into the media to rapidly activate VEGFR2.

To determine whether Ox-PAPC could bind to VEGFR2 and induce the phosphorylation at Tyr1175 we used HEK 293 cells stably expressing VEGFR2. Incubation with VEGF for 5 minutes induced the phosphorylation at Tyr1175 in these cells. However, incubation with Ox-PAPC for the same amount of time did not produce any change at this site (Figure 6a). Incubation of these cells with Ox-PAPC for longer periods of time up to 4 hours also did not show any change in phosphorylation (unpublished data, 2006). In addition, a reporter gene assay using an IL-8 promoter construct was transfected into HEK 293 cells expressing VEGFR2 and treated with VEGF or Ox-PAPC. VEGF was able to induce reporter activation in these cells whereas Ox-PAPC did not

Figure 4. Inhibition of VEGFR2 affects signal transduction pathways activated by Ox-PAPC. a, HAECs were incubated with or without 10 μmol/L SU-1498; then, 40 μg/mL Ox-PAPC (Ox) or media alone (C) was added to the cells for 4 hours. Equal amounts of protein were separated by gel electrophoresis and probed with antibodies raised against SREBP-1 (top). The ratio of mature to precursor form of SREBP is calculated for each treatment (bottom). Similar results were obtained in 3 independent experiments. b, HAECs were transfected with 5 nmol/L of siRNA VEGFR2 (siRNA) or 5 nmol/L of scrambled siRNA (SCR). After transfection cells were incubated with 30 μg/mL Ox-PAPC (Ox) or media alone (C) for 1 hour. After cell lysis, equal amounts of protein were separated by gel electrophoresis and probed for Erk 1/2 phosphorylation (Thr202/Tyr204) (top). The blot was then reprobed with Erk 1/2 for normalization (middle). The fold increase in phosphorylation induced by Ox-PAPC is calculated by comparison to the phosphorylation in cells treated with media alone (bottom). Similar results were obtained in 2 independent experiments. c, HAECs were incubated for 1 hour with or without 10 μmol/L SU-1498; then 40 μg/mL Ox-PAPC or media alone was added to the cells for 4 hours. Relative change in mRNA tissue factor (TF) were measured by qRT-PCR and normalized to GAPDH. Data represent mean±SD (n=3). **P<0.01 compared with cells treated without inhibitor using student t test.
induces the cleavage of SREBP-1 that results in a more sustained upregulation of IL-8 as well as the increase in LDL-R. This mechanism takes place through cholesterol depletion and eNOS uncoupling and is independent from the c-Src/STAT-3 pathway. Both of these pathways appear to be important in atherosclerosis as demonstrated by their activation in human lesions using immunohistochemistry. Our data indicate that Ox-PAPC activates SREBP-1 through VEGFR2 (Figure 4a). The ability of VEGFR2 to mediate the activation of SREBP and the upregulation of IL-8 and LDL-R has been shown by others to occur when endothelial cells are treated with VEGF. The upregulation of LDL-R mediated by VEGFR2 could promote further uptake of LDL and increase the amount of mmLDL in the subendothelial space.

The expression of TF can be induced by mm-LDL and Ox-PAPC. The upregulation of TF by Ox-PAPC takes place through two different pathways that result in the activation of the early growth response factor 1 (EGR-1) and the nuclear factor of activated T cells (NFAT). In addition, both pathways are activated by VEGF in the induction of TF. The activation of EGR-1 is Erk-dependent. Our results show that Erk 1/2 activation and increase in TF synthesis are downstream targets of the activation of VEGFR2 by Ox-PAPC (Figure 4b and 4c). Thus these studies provide evidence that VEGFR2 mediates effects of Ox-PAPC by stimulating specific signal transduction pathways.

A recent report indicated that lysophosphatidylcholine (LPC) activates cell proliferation in HUVECs by VEGFR2. LPC is present in ox-LDL and, at a much smaller extent, in mm-LDL and Ox-PAPC. However, LPC at concentrations found in Ox-PAPC was not able to activate transcription of IL-8. Among the components of Ox-PAPC, PEIPC is the most potent activator of inflammation, being active at concentrations as low as 100 ng/mL. These studies have also demonstrated that PEIPC activates the induction of IL-8 and LDL-R through VEGFR2 (Figure 2b).

We have performed studies to examine the mechanism of activation of VEGFR2 by Ox-PAPC. We have ruled out the possibility that Ox-PAPC rapidly induces the release of VEGF in endothelial cells that could lead to rapid autocrine stimulation. The presence of an antibody to VEGF was not able to reduce the induction of IL-8 by Ox-PAPC (Figure 5). We also have evidence that Ox-PAPC cannot independently activate VEGFR2 to cause its phosphorylation because VEGF, but not Ox-PAPC, led to phosphorylation of Tyr in HEK 293 cells expressing VEGFR2 (Figure 6a). Other investigators have described evidence for a ligand-independent activation of VEGFR2 by shear stress. This activation involves a complex formed by the platelet endothelial cell adhesion molecule (PECAM)-1 and VEGFR2, where PECAM-1 senses the fluid shear stress and VEGFR2 initiates the signaling. c-Src mediates the signal transduction between PECAM-1 and VEGFR2. Our studies suggest a c-Src–dependent, ligand-independent mechanism of activation of VEGFR2 by Ox-PAPC. This may be attributable to the interaction of Ox-PAPC with another surface molecule to activate VEGFR2. Other investigators have reported the derivatization of platelet-derived growth factor receptor β by 4-hydroxynonenal which results in phosphorylation of the receptor through an unknown mechanism. It is possible that

**Figure 5.** VEGF antibody does not inhibit Ox-PAPC induction of IL-8. HAECS were pre-incubated for 1 hour with different amounts of an antibody to VEGF (0, 1, 10, and 20 μg/mL). Afterward, 30 μg/mL Ox-PAPC, VEGF (10 and 50 ng/mL), or media alone were added to the cells and incubated for 4 hours. The media was collected and measured for IL-8 secretion by ELISA. Data represent mean ± SD (n=3). **P<0.01 compared with 0 μg/mL of VEGF antibody as calculated by one-way ANOVA.

(unfinished data, 2006). We finally examined whether activation of VEGFR2 takes place through a ligand-independent mechanism as it has been previously reported for shear stress. Inhibition of c-Src kinase with 5 μmol/L PP2 was able to reduce the phosphorylation of VEGFR2 at Tyr1175 in HAECS treated with Ox-PAPC (Figure 6b). siRNA to c-Src was also able to decrease the phosphorylation of VEGFR2 at Tyr1175 induced by Ox-PAPC (Figure 6c). Therefore, the activation of VEGFR2 by Ox-PAPC is, at least in part, c-Src kinase dependent.

**Discussion**

The current studies show an important role for VEGFR2 in the response of HAECS to Ox-PAPC and its component PEIPC. An exploratory survey that looked for changes in tyrosine phosphorylation of proteins by mass spectrometry indicated that Ox-PAPC induces the phosphorylation of VEGFR2 (unpublished data, 2005). The activation of VEGFR2 by Ox-PAPC is mediated through the phosphorylation of Tyr1175 (Figure 1). Phosphorylation of VEGFR2 at Tyr1175 is essential in endothelial cell development during embryogenesis, proliferation, and cell response to shear stress. In contrast to a less sustained activation of VEGFR2 observed for VEGF, the activation of VEGFR2 by Ox-PAPC was sustained for at least 4 hours after treatment. This extended activation of VEGFR2 has also been shown on stimulation of endothelial cells by shear stress.

Using a chemical inhibitor and siRNA we established a role for VEGFR2 in the transcription of IL-8, LDL-R, and TF induced by Ox-PAPC and PEIPC. These results do not exclude the possible involvement of VEGFR1 or VEGFR3 in the activation of endothelial cells by Ox-PAPC. There are at least 2 known signaling mechanisms that regulate IL-8 induction by Ox-PAPC: c-Src/STAT-3 and e-NOS/SREBP activation. Ox-PAPC and its components induce rapid phosphorylation of c-Src at Tyr1245 followed by the phosphorylation of STAT-3 at Tyr705, which translocates to the nucleus and causes an early upregulation of IL-8. Secondly, Ox-PAPC induces the cleavage of SREBP-1 that results in a more sustained upregulation of IL-8 as well as the increase in LDL-R. This mechanism takes place through cholesterol depletion and eNOS uncoupling and is independent from the c-Src/STAT-3 pathway. Both of these pathways appear to be important in atherosclerosis as demonstrated by their activation in human lesions using immunohistochemistry. Our data indicate that Ox-PAPC activates SREBP-1 through VEGFR2 (Figure 4a). The ability of VEGFR2 to mediate the activation of SREBP and the upregulation of IL-8 and LDL-R has been shown by others to occur when endothelial cells are treated with VEGF. The upregulation of LDL-R mediated by VEGFR2 could promote further uptake of LDL and increase the amount of mmLDL in the subendothelial space.

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the binding of Ox-PAPC to VEGFR2 in HAECs causes a change in configuration that would make this receptor susceptible to phosphorylation by c-Src which is activated by Ox-PAPC.

In summary, our data suggests that the activation of endothelial cells by Ox-PAPC at the early stages of the inflammation process involves VEGFR2. We demonstrate that after Ox-PAPC treatment, the increase of IL-8 (an inflammatory gene), LDL-R (sterol sensing), and TF (coagulation) are regulated, at least in part, by the activation of VEGFR2. We present evidence that this upregulation of transcription involves VEGFR2-mediated activation of Erk 1/2 and SREBP-1. Thus these data suggest that, together with PG E2 receptor subtype 2, VEGFR2 initiates signal transduction pathways that contribute to inflammatory conditions.

Acknowledgments
The authors thank Dr. Bruce I. Terman for providing HEK 293 cells transfected with VEGFR2. His numerous contributions to signaling in angiogenesis have enriched our understanding in the field.

Figure 6. Ox-PAPC induces the phosphorylation of VEGFR2 at Tyr1175 through a ligand-independent mechanism. a, HEK 293 stably expressing VEGFR2 were incubated with media alone (C), 30 μg/mL Ox-PAPC (Ox), or 50 ng/mL VEGF for 5 minutes. Equal amounts of protein were separated by gel electrophoresis and probed with an antibody raised against phospho-tyrosine VEGFR2 (Tyr1175) (top). The blot was then reprobed with VEGFR2 antibody for normalization (middle). The fold increase in phosphorylation induced by Ox-PAPC and VEGF is calculated by comparison to the phosphorylation in cells treated with media alone (bottom). Similar results were obtained in 2 independent experiments. b, HAECs were incubated with or without 5 μmol/L PP2; then, 30 μg/mL Ox-PAPC (Ox) or 30 μg/mL PAPC (P) were added to the cells for 2 hours. Equal amounts of protein were separated by gel electrophoresis and probed with antibodies raised against phospho-tyrosine VEGFR2 (Tyr1175) (top). The blot was then reprobed with VEGFR2 antibody for normalization (middle). The fold increase in phosphorylation induced by Ox-PAPC is calculated by comparison to the phosphorylation in cells treated with PAPC (bottom). Similar results were obtained in 3 independent experiments. c, HAECs were transfected with 5 nmol/L of siRNA c-Src (siRNA) or 5 nmol/L of scrambled siRNA (SCR); then, 30 μg/mL Ox-PAPC (Ox) or media alone (C) were added to the cells for 5 minutes. Equal amounts of protein were separated by gel electrophoresis and probed with antibodies raised against phospho-tyrosine VEGFR2 (Tyr1175) (top). The blot was then reprobed with VEGFR2 antibody for normalization (middle). The fold increase in phosphorylation induced by Ox-PAPC is calculated by comparison to the phosphorylation in cells treated with media alone (bottom). Similar results were obtained in 2 independent experiments.

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Disclosures
None.

References


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SUPPLEMENTAL DATA

EXPERIMENTAL PROCEDURES

Materials – Mouse anti-human VEGFR1 (clone BK302) was purchased from Upstate (Lake Placid, NY). Mouse anti-GAPDH was obtained from Chemicon (Temecula, CA).

qRT-PCR – Primers to measure relative changes in VEGF receptor 1 (VEGFR1) mRNA had the sequences: 5’AGGGCCTCTGATGTTGAATTGA3’ and 5’TAGGCTCCATGTGTAGTGCTGCAT3’. For VEGF receptor 2 (VEGFR2) mRNA the sequences were: 5’TCAGCTATGCTGGCATGTCTTCT3’ and 5’ATTCCATAGACGGACCTCAGAACCAC3’

For VEGF receptor 3 (VEGFR3) mRNA the sequences were:
5’AAAGGCTCTGACACCACAGACA3’ and 5’TGCCTGCTCTCTATCTGCTCAAACTC3’

Values were normalized to GAPDH.

FIGURES

Figure I. HAECs were incubated with increasing concentrations of Ox-PAPC (0 to 50 µg/ml) for 5 minutes. Equal amounts of protein were separated by gel electrophoresis and probed with an antibody raised against phospho-tyrosine VEGFR2 (Tyr1175) (top). The blot was then re-probed with VEGFR2 antibody for normalization (middle). The fold increase in phosphorylation induced by Ox-PAPC is calculated by comparison to the phosphorylation in cells treated with media alone (bottom).

Figure II. siRNA targeting VEGFR2 is able to specifically reduce the expression of VEGFR2 in HAECs. HAECs were transfected with 5 nM of siRNA VEGFR2 (siRNA) or 5 nM of scrambled siRNA (SCR). (a) One, two and four days post-transfection the cells were lysed. Equal amounts of protein were separated by gel electrophoresis and probed with an antibody raised against VEGFR2 (top), VEGFR1 (middle) and GAPDH (bottom). (b) Percentage changes in VEGFR1,
VEGFR2 and VEGFR3 mRNA were measured by qRT-PCR and normalized to GAPDH. Data represent mean±SD (n=3). **, p<0.01 compared with cells transfected with scrambled siRNA using student t test.
Online Figure I

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<th>µg/ml Ox-PAPC</th>
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![Graph showing normalized ratio of pY1175-VEGFR2 / VEGFR2 against µg/ml Ox-PAPC]
Online Figure II

a

<table>
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b

![Graph showing relative mRNA levels of VEGFR1, VEGFR2, and VEGFR3](image)

**Relative mRNA levels (% over SCR)**

VEGFR1 VEGFR2 VEGFR3

**Significance:** **

4