Role of Endothelial Nitric Oxide Synthase in the Regulation of SREBP Activation by Oxidized Phospholipids

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Abstract—Oxidized-1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (Ox-PAPC), found in atherosclerotic lesions and other sites of chronic inflammation, activates endothelial cells (EC) to synthesize chemotactic factors, such as interleukin (IL)-8. Previously, we demonstrated that the sustained induction of IL-8 transcription by Ox-PAPC was mediated through the activation of sterol regulatory element–binding protein (SREBP). We now present evidence for the role of endothelial nitric oxide synthase (eNOS) in the activation of SREBP by Ox-PAPC. Ox-PAPC treatment of EC induced a dose- and time-dependent activation of eNOS, as measured by phosphorylation of serine 1177, dephosphorylation of threonine 495, and the conversion of L-arginine to L-citrulline. Activation of eNOS by Ox-PAPC was regulated through a phosphatidylinositol-3-kinase/Akt-mediated mechanism. These studies also demonstrated that pretreatment of EC with NO donor, N\(^{\text{-}}\)-nitro-L-arginine-methyl ester (L-NAME), significantly inhibited Ox-PAPC–induced IL-8 synthesis. Because SREBP activation had been previously shown to regulate IL-8 transcription by Ox-PAPC, we examined the effects of L-NAME on Ox-PAPC–induced SREBP activation. Our data demonstrated that Ox-PAPC–induced SREBP activation and expression of SREBP target genes were significantly reduced by pretreatment with L-NAME. Interestingly, treatment of EC with NO donor, \(\text{S-nitroso-N-acetylpenicillamine}\), did not activate SREBP, suggesting that NO alone was not sufficient for SREBP activation. Rather, our findings indicated that superoxide (O\(_{2}^{\text{-}}\)), in combination with NO, regulated SREBP activation by Ox-PAPC. We found that Ox-PAPC treatment generated O\(_{2}^{\text{-}}\) via an eNOS-mediated mechanism and that mercaptoethylguanidine, a peroxynitrite scavenger, reduced SREBP activation by Ox-PAPC. Taken together, these findings propose a novel role for eNOS in the activation of SREBP and SREBP-mediated inflammatory processes. (Circ Res. 2006;98:0-0.)

Key Words: oxidized phospholipids ■ interleukin-8 ■ endothelium ■ nitric oxide ■ cholesterol

Atherosclerosis is a chronic inflammatory condition characterized by lipid accumulation and monocyte entry into the subendothelium. Previous work by our laboratory has demonstrated that phospholipid oxidation products of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (PAPC) accumulate in atherosclerotic lesions and other sites of chronic inflammation and activate endothelial cells (EC), leading to enhanced monocyte/EC interactions. Oxidized PAPC (Ox-PAPC) and its component phospholipids, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine (POVPC) and 1-palmitoyl-2-epoxyisoprostane E\(_{2}\)-sn-glycero-3-phosphorylcholine (PEIPC), also increase transcription of the atherogenic chemotactic factor, interleukin (IL)-8, in a sustained manner through an nuclear factor (NF)-\(\kappa\)B–independent pathway. In a recent article, we demonstrated that the sustained induction of IL-8 transcription by Ox-PAPC and its component phospholipids was mediated through a novel pathway, involving the activation of sterol regulatory element–binding protein (SREBP).

SREBP1s are a family of transcription factors that regulate cellular cholesterol homeostasis. These proteins are located in the endoplasmic reticulum in a loosely bound state, which acts as an inactive precursor to a lipoprotein. SREBPs then enter the nucleus and increase transcription of genes regulating cholesterol and fatty acid metabolism. Previously, we demonstrated that Ox-PAPC treatment of EC depleted caveolar cholesterol and activated both SREBP 1 and 2 within 30 minutes. SREBP activation by Ox-PAPC was maintained for at least 8 hours, and Ox-PAPC treatment also increased binding of SREBP to a novel SRE sequence in the IL-8 promoter. The current studies address the mechanism by which Ox-PAPC regulates SREBP activation. We provide evidence supporting a role for endothelial nitric oxide synthase (eNOS) in the regulation of SREBP activation and IL-8 transcription by Ox-PAPC. eNOS is a member of the family of nitric oxide synthases (NOs), which are responsible for the synthesis of NO from the metabolism of L-arginine to L-citrulline. In the normal...
endothelium, eNOS activity can be regulated by chemical agonists, including bradykinin\(^a\) and acetylcholine,\(^2\) and physical stimuli, such as shear stress.\(^10\) The subcellular localization of eNOS also plays an important role in its activity. Recent findings have demonstrated eNOS localization in the Golgi apparatus\(^11\) and in caveolar membranes;\(^12\) the latter appears to be an important compartment regulating eNOS-mediated NO production. Furthermore, phosphorylation of eNOS at serine residue 1177 (S1177) and dephosphorylation at threonine residue 495 (T495) have been associated with increased eNOS activity.\(^13\) Under certain pathological conditions, eNOS can also independently generate superoxide (O\(^2-\)).\(^14\) In the presence of O\(^2-\) generated by eNOS or other enzymes, NO can react to form peroxynitrite (ONOO\(^-\)). ONOO\(^-\) is a potent oxidizing agent that has been implicated to play a role in several inflammatory conditions, including atherosclerosis.\(^15\)

In the current studies, we demonstrate that Ox-PAPC treatment of EC activates eNOS in a dose- and time-dependent manner, while also inducing a concomitant increase in O\(^2-\) production. Furthermore, our findings demonstrate for the first time that eNOS activation regulates the activation of SREBP and the induction of SREBP target genes by oxidized phospholipids.

**Materials and Methods**

### Material and Reagents

Medium 199 for Human Aortic EC (HAEC) and DMEM medium for bovine aortic EC (BAEC) were purchased from Irvine Scientific. FBS was obtained from HyClone. Oxidized phospholipids were prepared as previously described.\(^2\) Rabbit polyclonal antibodies against eNOS S1177, eNOS T495, Akt S473, total Akt, and histone H2A were purchased from Cell Signaling. Rabbit polyclonal antibody against total eNOS, SREBP-1, and SREBP-2 were purchased from Santa Cruz Biotechnology. N\(^\text{6}\)-Nitro-L-arginine methyl ester (L-NAME), N\(^\text{6}\)-nitro-N-arginine methyl ester (D-NAME), methyl-beta-cyclodextrin (M\(\beta\)CD), mercaptoethylguanidine sulfate (MEG), and superoxide dismutase (SOD) were purchased from Sigma. S-Nitroso-N-acetylpenicillamine (SNAP), and N-acetyl-Leu-Leu-Norleucinal (ALLN) were purchased from Calbiochem. 2',7'-Dichlorofluorescein diacetate bis-acetoxymethyl ester (DCFH-DA) was purchased from Molecular Probes.

### Cell Culture and Treatment

HAEC (passages 4 to 8) were isolated and cultured as previously described.\(^16\) BAEC (passages 5 to 17) were purchased from VEC Technologies and cultured as previously described.\(^3\) Treatment with lipids and other activating agents were performed in media containing 1% to 2% FBS. For all inhibitor studies, cells were pretreated with inhibitor and subsequently cotreated with lipid and inhibitor for the indicated times.

### Enzyme-Linked Immunosorbent Assay

Secreted IL-8 levels in cell media were measured using the IL-8 ELISA kit (R&D Systems) according to the protocol of the manufacturer.\(^4\)

### Western Blot Analysis

Western blot analyses were performed as previously described.\(^5\) Blots were developed using enhanced chemiluminescence (ECL) plus (Amersham International) and levels were detected using the Bio-Rad VersaDoc Imaging System (Model 5000).

### Real-Time RT-PCR Using SYBR

RT-PCR was performed as previously described.\(^8\) Primer sequences were: IL-8, 5'-ttcttcggctgcctagtatttc-3', 5'-aacttggccgagaaagctgag-3'; LDLR, 5'-ctgctggctttctcaaaagtaa-3', 5'-aaagatgaggaagatacgcggtta-3'; Insig-1, 5'-ggacagactatagctatgggtt-3', 5'-gagctttggctaaagccgca-3'; and GAPDH, 5'-cattgcctcaagcaccaacctggtt-3', 5'-accaccctgtcgctgcagc-3'.

### Measurement of eNOS Activation

NO synthesis was determined in intact cells as previously described.\(^17\) Cells were incubated with 0.75 mCi/mL \(^3\)H-L-arginine for 30 minutes and then treated with lipids at the indicated concentrations for 30 minutes. To ensure that treatment did not affect the loading of the cells with \(^3\)H-L-arginine, the amount of \(^3\)H-L-arginine associated with the cells was determined. The cells contained 212 957±9128 DPM/well of \(^3\)H-L-arginine independent of the treatment and time.

### Measurement of Intracellular Oxidative Stress

Oxidation of DCFH, a nonfluorescent probe, to a green fluorescent product, 2',7'-dichlorofluorescein (DCF) was used to monitor intracellular oxidative stress. Following treatment, HAEC were rinsed with 1xPBS and DCFH-DA was added at a final concentration of 10 \(\mu\)mol/L and incubated for up to 30 minutes. Cells were rinsed again with 1x PBS and maintained in culture medium. Fluorescence was monitored using a Nikon fluorescence microscope (excitation, 488 nm; emission, 610 nm) equipped with a FITC filter.

### Measurement of Extracellular O\(^2-\) Production

O\(^2-\) production was measured using the cytochrome C reduction assay as previously described.\(^18\) HAEC were exposed to lipids for up to 4 hours using media containing 100 \(\mu\)mol/L acetylated-ferricytochrome C (Sigma). Supernatants were taken for absorbance measurements at 550 nm (Beckman 640 spectrophotometer). The specificity of reduction by O\(^2-\) was established by comparing reduction rates in the presence and absence of SOD (60 \(\mu\)g/mL). The corrected rates of O\(^2-\) release were calculated from SOD-inhibitable ferricytochrome C absorbance at 550 nm using the molar extinction coefficient (\(e=21,000\) mol/L\(^-1\) cm\(^-1\)).

### Statistical Analysis

Data for Western blots and RT-PCR were normalized to total protein or housekeeping genes, and fold induction was calculated with respect to normalized control, set at the baseline of 1. Computer-assisted statistical analyses were performed using Microsoft Excel 2000. All probability values were calculated using the Student t test. A value of P<0.05 was considered significant.

### Results

**Ox-PAPC and Its Component Phospholipid, POVPC, Activate eNOS**

Activation of eNOS correlates positively with phosphorylation at S1177 and dephosphorylation at T495.\(^13\) To determine whether Ox-PAPC treatment of HAEC alters eNOS phosphorylation, phospho-specific antibodies to eNOS residues S1177 and T495 were used. Ox-PAPC treatment increased S1177 phosphorylation within 10 minutes, and this phosphorylation was sustained for up to 4 hours (Figure 1A, top). Total levels of eNOS, as measured by the eNOS antibody at all time points following Ox-PAPC treatment, remained unchanged (Figure 1A, middle). Using densitometry to normalize eNOS S1177 phosphorylation to total eNOS levels, Ox-PAPC treatment was found to induce a 4-fold increase in eNOS phosphorylation at the 1-hour peak (Figure 1A, bottom). Our data demonstrated that phosphorylation of eNOS S1177 by Ox-PAPC was also dose dependent (Figure 1B). Further-
Figure 1. Ox-PAPC treatment of EC activates eNOS. HAEC were either untreated (control [C]) or treated with Ox-PAPC (Ox, 50 μg/mL) for the indicated times (A), at increasing concentrations for 1 hour (B), or with PAPC (50 μg/mL), Ox-PAPC (Ox, 50 μg/mL), and POVPC (20 μg/mL) for 1 hour (C). Cell lysates were collected, and Western blot analysis was performed analyzing eNOS S1177 phosphorylation (top). D, HAEC were either untreated (control [C]) or treated with Ox-PAPC (Ox, 50 μg/mL) for the indicated times, and phosphorylation of eNOS at T495 was analyzed by Western blot analysis (top). For A through D, total eNOS levels were also examined by Western blot analysis (middle), and densitometry was performed to normalize phospho-eNOS levels to total eNOS levels (bottom). eNOS activation, as measured by the conversion of 3H-arginine to 3H-citrulline, was also analyzed in HAEC untreated (Control) or treated with Ox-PAPC (25 μg/mL) (E) and in BAEC untreated (Control) or treated with Ox-PAPC (20 μg/mL) or POVPC (20 μg/mL) (F) for 30 minutes. For E and F, data represent mean±SD (n=3). *P<0.05 and **P<0.01, compared with untreated cells. All experiments were repeated at least 3 times.
more, treatment of HAEC with POVPc induced eNOS S1177 phosphorylation, whereas unoxidized PAPC had no effect (Figure 1C). In contrast, phosphorylation of eNOS at T495 was reduced following treatment with Ox-PAPC; this effect was observed within 10 minutes and was sustained for at least 4 hours (Figure 1D).

An important measure of eNOS activity is the conversion of L-arginine to L-citrulline. Our data demonstrated that treatment of HAEC with Ox-PAPC (25 μg/mL) for 30 minutes induced a significant increase in L-arginine to L-citrulline conversion (Figure 1E). We also observed a significant increase in L-arginine to L-citrulline conversion in BAEC, following treatment with both Ox-PAPC and POVPc (Figure 1F).

Ox-PAPC Treatment Activates eNOS Through a Phosphatidylinositol 3-Kinase/Akt-Mediated Mechanism, Independent of c-Src Kinase and PKA

Phosphorylation of eNOS at S1177 can be mediated through a phosphatidylinositol 3-kinase (PI3K) pathway, in which PI3K phosphorylates and activates Akt kinase, which in turn phosphorylates eNOS. To determine the role of the PI3K/Akt pathway in eNOS activation by Ox-PAPC, we first examined the activation state of Akt following Ox-PAPC treatment. Activation of Akt correlates positively with phosphorylation at S473. Using the phospho-specific antibody to Akt residue S473, we demonstrated that Ox-PAPC treatment of HAEC activated Akt within 5 minutes and that this activation was maintained for at least 1 hour (Figure 2A, top). Total levels of Akt, as measured by the total Akt antibody at all time points following Ox-PAPC treatment, remained unchanged (Figure 2A, middle). Following normalization to total Akt levels, Ox-PAPC treatment was found to induce a 5-fold increase in Akt phosphorylation at the 1-hour peak (Figure 2A, bottom).

We next examined the role of PI3K/Akt in eNOS activation by Ox-PAPC, using the specific PI3K/Akt inhibitor LY294002. Pretreatment of HAEC with LY294002 significantly reduced Ox-PAPC–induced eNOS S1177 phosphorylation (Figure 2B, top), without having any significant affects on total eNOS levels (Figure 2B, middle). Following normalization to total Akt levels, Ox-PAPC treatment was found to induce a 5-fold increase in Akt phosphorylation at the 1-hour peak (Figure 2A, bottom).

In addition to Akt, protein kinases such as c-Src kinase and protein kinase A (PKA) have also been demonstrated to regulate eNOS S1177 phosphorylation. Furthermore, we previously reported that Ox-PAPC treatment of HAEC activates both c-Src kinase and PKA. These studies, however, demonstrated that neither PP2 (c-Src inhibitor) nor RP-cAMP (PKA inhibitor) had any significant effects on Ox-PAPC–induced eNOS S1177 phosphorylation (Figure 2C).

Role of eNOS Activation in Ox-PAPC–Induced IL-8 Synthesis

To examine the role of eNOS activation in the induction of IL-8 expression by Ox-PAPC, we used the NOS chemical...
inhibitor L-NAME and its inactive d analog (D-NAME). As a competitive inhibitor for L-arginine binding to the NOS active site, L-NAME serves as a general inhibitor for all NOS family members. Through PCR analysis, however, we have determined that HAEC express neither inducible NOS nor neuronal NOS (data not shown). Our data demonstrated that pretreatment of HAEC with L-NAME reduced Ox-PAPC–induced IL-8 protein synthesis in a dose-dependent manner, whereas pretreatment of HAEC with D-NAME at similar concentrations had no effect (Figure 3A). Pretreatment of HAEC with L-NAME also significantly reduced the levels of IL-8 mRNA induced by Ox-PAPC (Figure 3B). Furthermore, the inhibitory effects of L-NAME on IL-8 synthesis were specific to Ox-PAPC, as L-NAME had no significant effects on LPS-induced IL-8 synthesis (data not shown).

To determine whether NO itself could induce IL-8 synthesis in HAEC, we used the NO donor (±)-SNAP. This compound emits NO immediately after solubilization in aqueous buffers. Treatment of HAEC with SNAP induced a dose-dependent increase in IL-8 protein (Figure 3C) and mRNA (Figure 3D) synthesis.

**Role of eNOS in Ox-PAPC–Induced SREBP Activation**

Previously, we demonstrated that the sustained induction of IL-8 by Ox-PAPC was mediated through the activation of SREBP.5 We, therefore, examined the role of eNOS in Ox-PAPC–induced SREBP activation. Activation of SREBP is mediated by a posttranslational cleavage in which the immature (125-kDa) protein is enzymatically truncated into a smaller mature isoform that enters the nucleus. SREBP activation, therefore, can be measured as a decrease in the levels of the immature isoform, as well as an increase in the nuclear translocation of the mature isoform. Our findings demonstrated that pretreatment of HAEC with 2.0 mmol/L L-NAME for 1 to 2 hours significantly inhibited the activation of SREBP in the cytoplasm by Ox-PAPC but not by the cholesterol-sequestering agent MβCD (Figure 4A). Similar results were observed in HAEC following pretreatment with 1.0 mmol/L L-NAME for 8 hours and 0.25 mmol/L L-NAME for 16 hours (data not shown). Furthermore, we observed that pretreatment of HAEC with 2.0 mmol/L L-NAME for 1 to 2 hours significantly reduced the nuclear translocation of SREBP induced by Ox-PAPC but not by MβCD (Figure 4B).

To confirm the effects of L-NAME on Ox-PAPC–induced SREBP activation, we next examined the effects of L-NAME on the induction of SREBP target genes low-density lipoprotein receptor (LDLR) and Insig-1 by Ox-PAPC. Whereas Ox-PAPC treatment of HAEC induced an approximate 12-fold increase in LDLR mRNA levels in the absence of inhibitor, pretreatment of HAEC with L-NAME, but not D-NAME, significantly reduced Ox-PAPC–induced LDLR

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**Figure 3.** eNOS activation and NO regulate the induction of IL-8 by Ox-PAPC. HAEC were pretreated for 1 hour with the indicated concentrations of L-NAME or D-NAME and then stimulated with control (C) media or Ox-PAPC (Ox, 50 μg/mL) for 4 hours. IL-8 protein synthesis, as measured by ELISA (A); and IL-8 mRNA synthesis, as measured by quantitative RT-PCR (B), were analyzed. HAEC were treated with the indicated doses of SNAP for 4 hours and IL-8 protein synthesis, as measured by ELISA (A), and IL-8 mRNA synthesis, as measured by quantitative RT-PCR (D), were analyzed. Data represent mean ± SD (n = 3). * denotes P < 0.05 and ** denotes P < 0.01, compared with Ox-PAPC treated cells in the absence of inhibitor in A and B and compared with untreated cells in C and D. All experiments were repeated at least 3 times.
mRNA levels (Figure 4C). L-NAME pretreatment did not, however, reduce the expression of LDLR mRNA induced by MβCD (data not shown). Furthermore, L-NAME pretreatment had similar effects on the induction of Insig-1 by Ox-PAPC (Figure 4D).

To determine whether NO itself could activate SREBP, we treated HAEC with SNAP and examined the expression of SREBP target genes. Surprisingly, treatment of HAEC with SNAP induced a dose-dependent decrease in LDLR mRNA levels (Figure 4E).

**Role of eNOS-Induced O2•− Production in SREBP Activation by Ox-PAPC**

We next examined whether Ox-PAPC treatment of HAEC would induce oxidative stress and increase the levels of reactive oxygen species (ROS). Our data demonstrated that treatment of HAEC with Ox-PAPC induced a significant increase in DCF fluorescence, a marker of intracellular oxidative stress, within 5 minutes, that was sustained for at least 30 minutes following treatment (Figure 5A); unoxidized PAPC had no significant effect on DCF fluorescence. Fur-
thermore, treatment of HAEC with Ox-PAPC and its component PEIPC significantly increased the rate of O$_2^•$ production, as compared with treatment with unoxidized PAPC (Figure 5B).

Because eNOS may generate O$_2^•$ under conditions in which its activity state is “uncoupled,” we examined the effect of L-NAME on Ox-PAPC–induced O$_2^•$ production. Our findings demonstrated that pretreatment of HAEC with L-NAME significantly reduced the rate of O$_2^•$ production induced by Ox-PAPC (Figure 5C). These findings suggested that O$_2^•$ production, in combination with NO, might regulate the activation of SREBP by Ox-PAPC. In the presence of O$_2^•$, NO will react to form ONOO$^•$, a highly reactive oxidizing agent with potent inflammatory properties. We, therefore, examined the role of ONOO$^•$ in Ox-PAPC–induced SREBP activation using the ONOO$^•$ scavenger, mercaptoethylguanidine (MEG). Our data demonstrated that pretreatment of EC with MEG significantly inhibited the activation of SREBP (Figure 5D) and the induction of LDLR transcription by Ox-PAPC (Figure 5E).
Discussion

In these studies, we have reported the following findings. (1) Ox-PAPC treatment of EC activates eNOS in a dose- and time-dependent manner. (2) eNOS activation by Ox-PAPC is mediated through a PI3K/Akt-dependent mechanism, and Ox-PAPC treatment activates Akt within 5 minutes. (3) Furthermore, eNOS activation plays a role in mediating the activation of SREBP and the induction of IL-8 by Ox-PAPC. (4) Finally, we present evidence that eNOS activation generates \( \mathrm{O}_2^- \) and that \( \mathrm{O}_2^- \), in combination with NO, is responsible for the activation of SREBP by Ox-PAPC.

Whereas previous findings have demonstrated that oxidized lipids inhibit the activation of eNOS by agonists, such as acetylcholine, our studies have examined the effect of Ox-PAPC treatment on the basal activity state of eNOS. We have demonstrated that Ox-PAPC treatment activates eNOS directly by increasing the conversion of \( \mathrm{L}-\mathrm{arginine} \) to \( \mathrm{L}-\mathrm{citrulline} \) (Figure 1). In addition, whereas activation of eNOS by some agonists requires an increase in intracellular calcium, we have obtained evidence that Ox-PAPC treatment of HAEC does not increase intracellular calcium levels (data not shown). Rather, activation of eNOS by Ox-PAPC is mediated through phosphorylation of S1177 (Figure 1), which has been reported to regulate eNOS activation in the absence of detectable calcium fluxes. \( \mathrm{S1177} \) phosphorylation by Ox-PAPC is mediated through the activation of PI3K/Akt (Figure 2). Interestingly, we previously demonstrated an important role for PI3K activation in Ox-PAPC-induced monocyte binding to EC. Taken together, these findings suggest an important role for PI3K in the initiation of inflammatory signaling events mediated by Ox-PAPC.

Importantly, these studies have established a novel role for eNOS in the activation of SREBP by Ox-PAPC. In a recent article, we demonstrated that treatment of EC with Ox-PAPC activates SREBP and increases transcription of SREBP target genes.5 We now demonstrate that pretreatment of EC with L-NAME significantly reduces the activation and nuclear translocation of SREBP, as well as the expression of SREBP target genes LDLR and Insig-1 induced by Ox-PAPC (Figure 4). Treatment of EC with NO alone (in response to SNAP), however, did not activate SREBP (data not shown) and, in fact, inhibited the expression of SREBP target genes (Figure 4). Rather, our findings indicated that regulation of SREBP activation by eNOS was mediated through eNOS-induced \( \mathrm{O}_2^- \) production (Figure 5). Because Ox-PAPC treatment induced phosphorylation of eNOS at S1177 and dephosphorylation at T495, as well as an increase in L-NAME-inhibitable \( \mathrm{O}_2^- \) production, we propose that Ox-PAPC treatment concurrently activates and “uncouples” eNOS. Sessa and colleagues have reported that dephosphorylation at T495 may be an intrinsic switch mechanism controlling the production of \( \mathrm{O}_2^- \), rather than NO, by eNOS.\(^{50}\) Their studies demonstrated maximal \( \mathrm{O}_2^- \) production in cells where S1177 was constitutively phosphorylated and T495 was dephosphorylated.\(^{50}\) Previously, it has also been reported that eNOS uncoupling may occur during states in which eNOS is displaced from caveolae.\(^{14}\) We recently reported that Ox-PAPC treatment of EC disrupts caveolae and redistributes caveolin-1, a major structural protein present in caveolae.\(^{5}\) \( \mathrm{O}_2^- \) can readily combine with NO to form ONOO\(^-\) and it may also catalyze the formation of other hydroperoxides.\(^{31}\) The current studies have demonstrated that MEG, a scavenger of ONOO\(^-\), inhibits the activation of SREBP by Ox-PAPC (Figure 5). Taken together, these findings suggest that both NO and \( \mathrm{O}_2^- \) production play an important role in regulating SREBP activation by Ox-PAPC.

We propose the following potential mechanisms for the role of eNOS in SREBP activation by Ox-PAPC. First, the formation of ROS generated by eNOS, including \( \mathrm{O}_2^- \) and/or ONOO\(^-\), may alter caveolar membranes and regulate the depletion of cellular cholesterol induced by Ox-PAPC. Radi et al have demonstrated that ROS can alter the integrity of cell membranes via lipid peroxidation.\(^{31}\) Peterson et al have demonstrated that ROS can decrease caveolae number and oxidize cholesterol in caveolar membranes, which may result in cholesterol efflux from caveolae.\(^{32}\) Cholesterol oxidation has also been shown to redistribute caveolin from caveolar compartments to internal stores.\(^{33}\) Second, ROS may modify enzymes that regulate SREBP activation, such as SREBP cleavage-activating protein (SCAP) and/or the S1/S2 proteases. Several investigators have demonstrated that ROS, including ONOO\(^-\), can regulate the activation of proteases via nitrosylation of cysteine and/or tyrosine residues.\(^{35}\) One or both of the mechanisms may be important in mediating SREBP activation by Ox-PAPC.

In the current studies, we have also established a role for eNOS activation in the induction of IL-8 transcription by Ox-PAPC. We have demonstrated that pretreatment of EC with L-NAME inhibits Ox-PAPC–induced IL-8 expression (Figure 3). Although the effect of L-NAME on IL-8 expression may be attributable to the inhibitory action of L-NAME on Ox-PAPC–induced SREBP activation, it is important to note that eNOS can induce IL-8 expression via SREBP-independent mechanisms. Pae et al have demonstrated that treatment of EC with NO donor SNAP induces IL-8 synthesis via expression of vascular endothelial growth factor (VEGF).\(^{36}\) Furthermore, we have presented evidence that treatment with SNAP induces IL-8 transcription in HAEC (Figure 3). Therefore, we hypothesize that eNOS activation by Ox-PAPC may regulate IL-8 transcription through SREBP-dependent and SREBP-independent mechanisms. Because we previously reported a role for SREBP in the sustained induction of IL-8 transcription by Ox-PAPC, we propose that eNOS also regulates the sustained induction of IL-8 transcription by Ox-PAPC. This is in contrast with the c-Src/signal transducer and activator of transcription (STAT) 3 pathway, which we previously reported to regulate the early induction of IL-8 transcription by Ox-PAPC.\(^{24}\) In summary, our current model for the sustained activation of SREBP and induction of IL-8 transcription by Ox-PAPC suggests that Ox-PAPC treatment activates Akt, which promotes the activation of eNOS and the production of NO and \( \mathrm{O}_2^- \). Subsequently, \( \mathrm{O}_2^- \) and/or ONOO\(^-\), through a mechanism mediated by either cholesterol oxidation and depletion or enzymatic activation, activate SREBP, which translocates to the nucleus, binds to the SRE in the IL-8 promoter, and increases transcription of IL-8 (Figure 6).

Atherosclerosis is a chronic inflammatory condition characterized by endothelial dysfunction and lipid accumulation...
Previously, we demonstrated that Ox-PAPC treatment of EC (1) activates eNOS phosphorylation, thus perpetuating the cycle. In summary, these studies indicate superoxide.

**References**


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