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A role for NADPH oxidase 4 in the activation of vascular endothelial cells by oxidized phospholipids

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Abstract

Previous studies from our group have demonstrated that oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycerol-3-phosphocholine (Ox-PAPC) activates over 1000 genes in human aortic endothelial cell (HAEC). Prominent among these are genes regulating inflammation, cholesterol homeostasis, antioxidant enzymes, and the unfolded protein response. Previous studies from our lab and others suggested that transcriptional regulation by Ox-PAPC may be controlled, at least in part, by reactive oxygen species (ROS). We now present evidence that Ox-PAPC activation of NADPH oxidase 4 (NOX4) is responsible for the regulation of two of these important groups of genes: those controlling inflammation and sterol regulation. Our data demonstrate that Ox-PAPC increases reactive oxygen species formation in HAEC as seen by DCF fluorescence. NOX4 is the major molecule responsible for this increase since downregulation of NOX4 and its components (p22^{phox} and rac1) blocked the Ox-PAPC effect. Our data show that Ox-PAPC did not change NOX4 transcription levels but did induce recruitment of rac1 to the membrane for NOX4 activation. We present evidence that vascular endothelial growth factor receptor 2 (VEGFR2) activation is responsible for rac1 recruitment to the membrane. Finally, we demonstrate that knockdown of NOX4 and its components rac1 and p22^{phox} decrease Ox-PAPC induction of inflammatory and sterol regulatory genes, but do not affect Ox-PAPC transcriptional regulation of other gene of antioxidant and unfolded protein response. In summary, we have identified a VEGFR2/NOX4 regulatory pathway by which Ox-PAPC controls important endothelial functions.

Keywords

Ox-PAPC; NOX4; VEGFR2; atherosclerosis; reactive oxygen species; endothelium

Introduction

Oxidized phospholipids that contain modified arachidonic acid at the *sn*2-position accumulate in various chronic inflammatory sites including atherosclerotic lesions [1,2]. We demonstrated

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that oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycerol-3-phosphocholine (Ox-PAPC) regulates the expression of more than 1000 genes in the human aortic endothelial cell (HAEC) [3]. Many of these genes are also regulated by the most active component of Ox-PAPC: PEIPC (1-palmitoyl-2-(5,6-epoxyisoprostane E2)-*sn*-glycerol-3-phosphocholine) [4]. The regulated genes can be grouped into 13 modules, representing specific signaling pathways including inflammation, coagulation, sterol regulation, unfolded protein response, and redox signaling. Among them, the proinflammatory cytokines IL-8 and MCP-1, are significantly upregulated by Ox-PAPC, inducing monocyte recruitment and retention in the vessel wall [5]. The genes regulating sterol synthesis likely play an important role in determining the level of LDL transport into the vessel wall. Ox-PAPC was also shown to activate vascular endothelial growth factor receptor 2 (VEGFR2), and this activation led to the induction of IL-8 and caused SREBP activation, which increased expression of downstream targets such as LDLR [6]. However, the mechanism by which VEGFR2 activation regulated these genes was not identified.

The current study examined the role of NADPH oxidase 4 (NOX4) in Ox-PAPC regulation of proinflammatory and sterol regulatory genes. NOX4 is expressed abundantly in the vasculature [7,8] and previous studies have shown that superoxide ($O_2^{\bullet-}$), or its derivatives, including hydrogen peroxide (H_2O_2), can regulate gene expression by interaction with various transcriptional factors [9]. Previous reports showed that oxidized LDL and its bioactive component Ox-PAPC induced reactive oxygen species (ROS) formation in bovine and human endothelium [7,10]. However, the major source of ROS in HAEC formed in response to Ox-PAPC was not identified. The current study provides evidence that NOX4 is a major source of ROS produced in response to Ox-PAPC in HAEC.

The properties of NOX2 in regulating superoxide synthesis have been well-defined in phagocytic cells [11,12]. In the resting state, NOX2 and p22^{phox} form a complex, but upon activation, cytosolic regulatory components (p47^{phox}, p67^{phox}, rac) are recruited to form a functional complex. Currently, seven NOX subtypes have been identified, namely NOX1–5 and Duox1/2 [8,13,14]. Additional cytosolic regulatory components (p40^{phox}, NOXO1, NOXA1, DuoxA1/2) also have been identified [13,15]. The regulatory components of NOX4 have not been identified clearly, but previous reports suggested that p22^{phox} constitutively forms a complex with NOX4, and rac1 is required for NOX4 activation [16,17]. NOX4 is located in the perinuclear area in endothelial cells, suggesting a role in regulating gene expression in the nucleus [18].

In the current study, we demonstrate using gene silencing techniques that NOX4 is an important regulator of proinflammatory and sterol synthetic genes in HAEC and that VEGFR2 regulates this pathway.

Experimental Procedures

Materials and reagents

1-palmitoyl-2-arachidonyl-*sn*-glycerol-3-phosphocholine (PAPC) was purchased from Avanti Polar lipids and was oxidized by exposure to air for 48 hrs. The composition of Ox-PAPC was analyzed by electrospray ionization-mass spectrometry (ESI-MS) [19]. GAPDH, p22^{phox}, and rac1 antibodies were purchased from Cell Signaling. NOX4 antibody was from Lifespan BioSciences. PMSF, protease, and phosphatase inhibitors were purchased from Sigma. SU1498 were purchased from Calbiochem. 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) was purchased from Molecular Probes.

Cell culture

HAECs were isolated and maintained as described in core facility in University of California-Los Angeles (UCLA) atherosclerosis research unit [20,21]. Cells with 4–8 passages were used for experiments. Unless indicated, cells were pretreated with inhibitors for 1 hr and co-treated with stimuli for the indicated times. Human monocytes were isolated and differentiated to macrophages in the core facility in UCLA atherosclerosis research unit.

Gene silencing by siRNA-mediated transfection

NOX4 siRNAs (Hs_NOX4_1 HP and Hs_NOX4_2 HP), rac1 siRNA (Hs_RAC1_6), were purchased from Qiagen. Customized siRNAs of p22^{phox} (5'-CGC UGC AUU UAU UGC AGG UGG GUG CAC-3') and VEGFR2 (5'-AAU ACU UGU CGU CUG AUU CUC CAG G-3') were purchased from Integrated DNA technologies. Fluorescently-labeled control siRNA (GLO-siRNA) for the measurement of the efficiency of transfection was purchased from ThermoScientific. HAECs at 90% confluence were transfected with complex of siRNA (final concentration, 40nM) and Lipofectamine 2000 (Invitrogen) for 4 hr as recommended by manufacturer, and the complex solution was replaced with normal growth media. After 2–3 days of cell growth, gene silencing was determined by QRT-PCR and Western blotting.

Quantitative RT-PCR (QRT-PCR)

Total RNA and cDNA were prepared using RNA extraction kit (Qiagen or Bio-Rad) and iScript™ cDNA synthesis kit (Bio-Rad). SYBR® green master mixture and PCR amplification system (Applied Biosystems) were used for quantitative PCR. The transcriptional level of GAPDH was measured for normalization. The sequences of primer sets were; **GAPDH**, F:5'-CCT CAA GAT CAT CAG CAA TGC CTC CT-3', R:3'-GGT CAT GAG TCC TTC CAC GAT ACC AA-5'; **NOX1**, F:5'-CAA TCT CTC TCC TGG AAT GGC ATC CT-3', R:5'-CCT GCT GCT CGG ATA TGA ATG GAG AA-3'; **NOX2**, F:5'-AAG GCT TCA GGT CCA CAG AGG AAA-3', R:5'-AGA CTT TGT ATG GAC GGC CCA ACT-3'; **NOX3**, F:5'-ACC GTG GAG GAG GCA ATT AGA CAA-3', R:5'-CAG GTT GAA GAA ATG CGC CAC GAT-3'; **NOX4**, F:5'-AGC AGA GCC TCA GCA TCT GTT CTT-3', R:5'-TGG TTC TCC TGC TTG GAA CCT TCT-3'; **NOX5**, F:5'-CCT CCT CAT GTT CAT CTG CTC CAG TT-3', R:5'-AGG AGG TAG GAC AGG TGA GTC CAA TA-3'; **Duox1**, F:5'-TGT ATG TCT TTG CCT CCC ACC ACT-3', R:5'-TTG CTG GGA CCA GGA AGA AGA TGT-3'; **Duox2**, F:5'-AGT ACA AGC GCT TCG TGG AGA ACT-3', R:5'-TCT GCA AAC ACG CCA ACA CAG ATG-3'; **NOXA1**, F:5'-ACC ATG ATG CCA GGT CCC TAA TCA-3', R:5'-AGA GAG GAG CCT GTT TGC CAA CTT-3'; **NOXO1**, F:5'-AAT TCA GGC AGC TCA AGA CCC TCA-3', R:5'-ACA GTG GTG CAT CGA GAA GCT TTG-3'; **DuoxA1**, F:5'-AAG AAA GGA GGG CAT GTG GAG TCA-3', R:5'-TGT AAT CCC AGC ACT TTG GGA GGT-3'; **DuoxA2**, F:5'-TCA GCG TTC CAC TGC TCA TCG TTA-3', R:5'-TAC CCA CGA ACC ATT CTG CAC TGA-3'; **p47^{phox}**, F:5'-TAC GTG TTC TAT AGA GCC TGG CGT-3', R:5'-AGC AAC ATT TAT TGA GGG TGG CGG-3'; **p67^{phox}**, F:5'-GGC AAG CTG TTT CGA CCA AAT GAG AG-3', R:5'-ACC CAG AGA AAC TGT CTT GAT CCA CC-3'; **IL-8**, F:5'-ACC ACA CTG CGC CAA CAC AGA AAT-3', R:5'-TCC AGA CAG AGC TCT CTT CCA TCA GA-3'; **LDLR**, F:5'-CGT GCT TGT CTG TCA CCT GCA AAT-3', R:5'-AGA ACT GAG GAA TGC AGC GGT TGA-3'; **MCP-1**, F:5'-GAC ACT TGC TGC TGG TGA TTC TTC-3', R:5'-TGC TCA TAG CAG CCA CCT TCA TTC-3'; **ATF3**, F:5'-TTG CAG AGC TAA GCA GTC GTG GTA-3', R:5'-ATG GTT CTC TGC TGC TGG GAT TCT-3'; **HO-1**, F:5'-ATA GAT GTG GTA CAG GGA GGC CAT CA-3', R:5'-GGC AGA GAA TGC TGA GTT CAT GAG GA-3'; **rac1**, F:5'-TGC AGT TAG GAG GTG CAG ACA CTT-3', R:5'-CTG GTG AGT TCA ATG GCA ACG CTT-3'.

Western blotting

Laemmli buffer (2x, Bio-rad) containing protease and phosphatase inhibitor cocktails, and PMSF (1mM) was used for total cell lysate preparation [22]. The protein samples were separated on 4–20% Tris-glycine SDS gels (ISC Bioexpress). Standard protocol for SDS-PAGE electrophoresis and blotting were used as described [10]. Blots were incubated with primary and secondary antibodies, developed, and analyzed using enhanced chemiluminescence kit (Amersham) and VersaDoc Imaging System-Model 5000 (Bio-Rad). Quantity One® program was used to calculate band densities from the images.

Isolation of the membrane fractions

Cells were resuspended in buffer (10mM HEPES, 40mM KCl, 5mM MgCl₂, 1mM, EDTA) containing protease and phosphatase inhibitor cocktails, and PMSF (1mM). Cells were disrupted by 10 passages through a 26 gauge needle, followed by freezing and thawing. Lysates were centrifuged for 10min at 4000rpm. The supernatant was centrifuged for 1hr 15min at 35,000rpm. The final membrane pellets were resuspended in RAL buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2.5 mM MgCl₂, 1% NP-40, 10% glycerol) containing protease and phosphatase inhibitor cocktails, and PMSF (1mM).

Detection of intracellular reactive oxygen species (ROS) formation

CM-H₂DCFDA is a cell-permeant indicator for reactive oxygen species that is nonfluorescent until removal of the acetate groups by intracellular esterases and oxidation within the cell [23–25]. Cells were grown on glass chamber slides to confluence, and preincubated with CM-H₂DCFDA (5uM) for 30min in assay media (M199 plus 1% FBS) at 37°C in CO₂ incubator. After removing the CM-H₂DCFDA solution, cells were treated with stimuli and the green fluorescence (excitation: 514nm, detection: 560–615nm) was observed under inverted confocal microscopy with one minute intervals for the indicated time. The PASCAL program was used for scanning and imaging the signal. Minimal laser exposure was employed to prevent nonspecific bleaching of the cells during the observation. Identical laser intensity and settings were used for comparative measurements.

Statistical analysis

Two-tailed unpaired student t-test was used to evaluate the difference between two groups. P value <0.05 was regarded as a statistically significant.

Results

NOX4 is a major source of intracellular ROS formation induced by Ox-PAPC in HAEC

Previous studies using NOX inhibitor apocynin and NOX4 silencing suggested a role for NOX4 in Ox-PAPC-mediated ROS production in bovine aortic endothelial cells [7]. However, the NOX subtypes and the subunits regulating ROS formation were not identified. In HAEC, NOX4 is the major NOX subtype as determined by QRT-PCR (Table 1A–B). Other NOX subtypes and cytosolic regulatory molecules were detectable, but the expression levels for all except p22^{phox} were extremely low. In contrast, in human macrophage, NOX2, p22^{phox}, p47^{phox}, and p67^{phox} were abundantly expressed as reported [8] (Table 1C–D). To confirm that NOX4 is a major source of superoxide in HAEC, we employed a DCF assay, which measures intracellular ROS formation including hydrogen peroxide (H₂O₂) and peroxynitrite (ONOO⁻). Superoxide (O₂^{•-}) is rapidly converted to H₂O₂ by superoxide dismutase, or to ONOO⁻ by nonenzymatic interaction with NO from endothelial nitric oxide synthase (eNOS) [26]. To test the effects of NOX4 and its components in ROS formation, NOX4, p22^{phox}, and rac1 silencing were employed. Silencing of NOX4, p22^{phox}, and rac1 were confirmed by Western blotting (Figure 1A–C). We confirmed 100% efficiency of siRNA transfection in

HAECs using fluorescently-labeled control siRNA (GLO-siRNA) (Figure 1D). As shown in Figure 1E, Ox-PAPC (50ug/ml) significantly increased ROS formation in HAEC within 20 min; this increase was sustained for over 1hr. NOX4, rac1, and p22^{phox} gene silencing all significantly reduced the DCF signal induced by Ox-PAPC. The quantification of the DCF signal from 10 randomly chosen cell areas is shown in Figure 1F. These results suggest that the NOX4 is the major source of intracellular ROS formation induced by Ox-PAPC in HAEC, and regulatory components p22^{phox} and rac1 are required for NOX4 function [7]. In contrast to BAEC, Ox-PAPC (50ug/ml for 4hr incubation) did not change the level of NOX4 mRNA in HAEC (data not shown). The rapid formation of ROS in response to Ox-PAPC without significant change in NOX4 mRNA levels, strongly suggests that Ox-PAPC induced ROS formation is not by a change in NOX4 transcription.

NOX4 and p22^{phox} silencing downregulated proinflammatory and sterol regulatory gene induction by Ox-PAPC

To examine the role of the NOX4 complex in Ox-PAPC regulated gene expression, we tested the effect of NOX4 silencing on the induction of IL-8, MCP-1, and LDLR by Ox-PAPC. As shown in Figure 2A, NOX4 gene silencing significantly downregulated IL-8, MCP-1, and LDLR induction by Ox-PAPC. The levels of the other NOX subtypes were extremely low in HAEC (Table 1A). Interestingly, NOX4 gene silencing did not decrease Ox-PAPC induction of HO-1 or ATF3, which are representative Ox-PAPC-inducible genes involved in redox signaling and the unfolded protein response (UPR) pathways (Figure 2B). Because the expression level of p22^{phox} was relatively high (Table 1B) and a requirement of p22^{phox} for NOX4 action has been previously demonstrated [16], we tested the effect of silencing p22^{phox} on IL-8 gene induction. As shown in Figure 2C, p22^{phox} silencing caused a significant decrease in IL-8 induction by Ox-PAPC. Since NOX4 silencing did not completely inhibit IL-8 induction, we examined possible compensatory upregulation of other NOX subtypes by NOX4 silencing. However, NOX4 silencing did not induce any compensatory upregulation of other NOX subtypes (Figure 2D). This result suggested that NOX4 regulates a subset of genes controlled by Ox-PAPC.

Rac1 activation by Ox-PAPC controls IL-8 gene induction

Previous reports in other cell systems and with other cell treatments showed that an increase in mRNA levels of NOX4 was an important factor for the regulation of NOX4 function [8, 27–29]. However, Ox-PAPC (50ug/ml for 4hr incubation) did not increase NOX4 mRNA levels in HAEC (data not shown). This result suggested the presence of another regulatory mechanism for NOX4 function by Ox-PAPC in HAEC. Rac1 transcript is abundant in HAEC and rac1 is an important regulatory component for the activation of NOX4 [17]. In untreated cells, rac1 is primarily found in the cytosol; however, upon activation, rac1 is recruited to the plasma membrane where it can interact with various signaling molecules, including NOX4 [30]. Therefore, the relative content of rac1 in the membrane fractions was used as a measure of rac1 activation [31]. As shown in Figure 3A, Ox-PAPC (50ug/ml) induced rac1 recruitment to the cell membrane after 10min, and this recruitment was sustained up to 4hrs. The same blot was reprobed with caveolin-1 antibody as a loading control. The silencing of rac1 using siRNA transfection decreased IL-8 induction in HAEC (Figure 3B), suggesting that rac1 regulates both ROS formation (Figure 1E–F) and inflammatory gene expression through the activation of NOX4.

VEGFR2 is involved in rac1 activation by Ox-PAPC in HAEC

Previously, we reported that vascular endothelial growth factor receptor 2 (VEGFR2) is activated by Ox-PAPC and mediates Ox-PAPC-induced IL-8 and LDLR expression in HAEC [6]. Inhibition of VEGFR2 by SU1498 and VEGFR2 siRNA transfection decreased Ox-PAPC

and PEIPC regulation of IL-8 and LDLR [6]. We showed that SU1498 almost completely blocked rac1 recruitment to the cell membrane by Ox-PAPC, suggesting that VEGFR2 is an upstream regulator of rac1 and subsequent NOX4 activation by Ox-PAPC in these cells (Figure 4A). The silencing of VEGFR2 (Figure 4B) also resulted in a significant reduction of rac1 translocation to the membrane by Ox-PAPC (Figure 4C).

Discussion

Our previous report showed that treatment of HAEC for 4 hours with Ox-PAPC regulated the expression of approximately 1000 genes involved in at least 13 different aspects of endothelial cell function, including the proinflammatory cytokines IL-8 and MCP-1 and the sterol regulatory gene LDLR [3]. Ox-PAPC also increased reactive oxygen species (ROS) formation in the vascular endothelium [4,7,10]. Previous reports have highlighted the role of ROS in regulating gene transcription in the vascular systems by interactions with signaling molecules including transcriptional factors [9]. In the current study, we focused on the role of NOX in producing ROS that regulate gene expression in endothelial cells because NOX has been reported to be the primary source of ROS in the vascular system [32]. We observed that in HAEC, amongst seven NOX subtypes, NOX4 was the only highly expressed subtype (Table 1). As shown in Figure 1, the importance of NOX4 in regulating ROS production in HAEC treated with Ox-PAPC was demonstrated by the reduction of ROS formation after silencing of NOX4 or its components rac1 and p22^{phox}. As opposed to the previous report in BAEC [7], this current study of Ox-PAPC did not induce NOX4 up-regulation in HAEC, demonstrating that transcription is not a factor for determining NOX4 activity in the action of Ox-PAPC. Rather we present evidence that a regulatory mechanism through rac1 translocation is involved.

VEGFR2 was suggested to be an upstream regulator of the NOX2 complex in different experimental systems [33,34]. Our studies suggest for the first time that VEGFR2 is an upstream regulator of NOX4 in HAEC, and that activation of VEGFR2 by Ox-PAPC causes rac1 activation and subsequent NOX activation. The exact mechanism of rac1 activation by VEGFR2 in HAEC is not known clearly, but previous reports suggested that the phosphorylation Tyr¹²¹⁴ of VEGFR2 induces the recruitment of adaptor protein Shb to the receptor and mediates PI3K-IQGAP1-rac1 pathway [30,35,36]. A similar role of epidermal growth factor receptor (EGFR) was also shown in the activation of rac1-NOX2 complex in vascular smooth muscle cells [37].

To determine the role of NOX4 in pro-inflammatory gene induction by Ox-PAPC, we tested the effects of silencing of NOX4 and its components p22^{phox} and rac1 on IL-8 regulation by Ox-PAPC. Silencing of NOX4, p22^{phox}, and rac1 caused a significant decrease of IL-8 induction by Ox-PAPC. Previously, we reported that Ox-PAPC activates eNOS to increase the formation of reactive oxygen species (e.g. ONOO⁻) in HAEC and that ROS are responsible for the induction of IL-8 and LDLR [10]. From the results of the current study, we suggest that the superoxide from NOX4 is rapidly converted to peroxynitrite (ONOO⁻) by diffusion-controlled interaction with NO derived from eNOS to regulate IL-8 and LDLR expression in HAEC. We also confirmed that classical NOX inhibitors (apocynin and diphenyleneiodonium) showed efficient inhibition of IL-8, MCP-1 and LDLR expression by Ox-PAPC (data not shown). However, since previous reports showed that DPI and apocynin are not specific to the NOX complex [38–41], we employed the silencing of NOX4 and its components to address their role in gene regulation in HAEC.

Interestingly, while NOX4 gene silencing downregulated IL-8, MCP-1 and LDLR, NOX4 silencing did not inhibit the induction of HO-1 or ATF3, suggesting NOX4 is involved in specific proinflammatory signaling pathways in HAEC. Previously, we reported that Ox-PAPC induced pro- and anti-atherogenic responses in HAEC through separate signaling pathways

[4,6,42]. The blocking of VEGFR2 signaling or co-treatment of high-density lipoprotein (HDL) decreased proinflammatory gene (IL-8) induction by Ox-PAPC. However, the induction of HO-1 by Ox-PAPC was not affected by VEGFR2 inhibition or HDL co-treatment. Thus, our results suggest that NOX4 is involved in mediating pro-atherogenic responses but not in mediating anti-atherogenic responses in HAEC. Recently, we reported that another redox regulating system, plasma membrane electron transport (PMET), is involved in the regulation of HO-1 transcription, but not in the regulation of IL-8 and LDLR, by Ox-PAPC in endothelial cells [43].

In conclusion, we demonstrated that Ox-PAPC regulates proinflammatory and sterol regulatory genes (IL-8, MCP-1, and LDLR) through NOX4 activation. We also demonstrated that VEGFR2-mediated rac1 activation is involved in the regulation of NOX4 activation and downstream gene regulation in HAEC.

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List of Abbreviations

ATF-3	activating transcription factor 3
BAEC	bovine aortic endothelial cells
DCF	dichlorofluoreceine
eNOS	endothelial nitric oxide synthase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HAEC	human aortic endothelial cell
HO-1	heme oxygenase-1
IL-8	interleukin-8
LDLR	low-density lipoprotein receptor
MCP-1	monocyte chemotacting protein-1
NOX	NADPH oxidase
PEIPC	1-palmitoyl-2-(5,6-epoxyisoprostane E2)- <i>sn</i> -glycero-3-phosphocholine

Ox-PAPCoxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycerol-3-phosphocholine**UPR**

unfolded protein response

VEGFR2

vascular endothelial growth factor receptor type 2

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FIG. 1. NOX4 complex is an important source of intracellular reactive oxygen species (ROS) formation in response to Ox-PAPC

(A–C) HAECs were transfected with scrambled (sc), NOX4, p22^{phox}, and rac1-specific siRNAs (NOX4si, p22si, rac1si) for gene silencing. After 2 days, protein levels of NOX4, p22^{phox} and rac1 were determined. (D) To examine the efficiency of siRNA transfection in HAECs, the scrambled- (20nM) and fluorescently-labeled siRNA (GLO-siRNA, 20nM) were transfected into HAECs using Lipofectamine 2000 reagent. After 24hr incubation, the presence of fluorescently-labeled GLO-siRNA in the cells was confirmed by detecting fluorescence intensity (514nm wavelength for excitation and 560–615nm wavelength for detection, resolution:100x). (E) After 2 days of siRNA transfection, intracellular ROS formation in response to Ox-PAPC (50ug/ml, Ox50) or media (C) were measured by DCF assay as described in the method section. The image shown is the relative fluorescence intensities at 0, 20min points of Ox-PAPC treatment. (F) Quantification of the DCF signal by measuring mean fluorescence intensities of 10 randomly chosen cell area from the images in (D). Values are represented as mean $\pm$ SEM, *** $p < 0.001$; n

FIG. 2. NOX4 and p22^{phox} gene silencing downregulated induction of pro-inflammatory and sterol synthetic genes by Ox-PAPC but not redox- and UPR-regulating genes

(A) HAECs were transfected with scrambled (sc) or NOX4-specific siRNA (NOX4 si) as described in the method section. After 2 days of cell growth, cells were treated Ox-PAPC (50 μ g/ml, Ox50) or media (C) for 4 hrs, and relative mRNA levels of proinflammatory and sterol synthetic genes (IL-8 MCP-1, LDLR) were determined by QRT-PCR. (B) Transcriptional levels of other NOX subtypes were measured by QRT-PCR to examine the presence of compensatory up-regulations of other NOX subtypes by NOX4 silencing in HAEC. (C) HAECs were transfected with scrambled (sc) or siRNA to p22^{phox} (p22 si), and after 2 days the expression of IL-8 by Ox-PAPC (50 μ g/ml) was measured by QRT-PCR. (D) HAECs were transfected with NOX4 siRNA and treated with Ox-PAPC as in (A), and the transcriptional levels of HO-1 and ATF3 were determined by QRT-PCR. Values are mean $\pm$ SEMs of triplicate PCR reactions; *** p<0.001.

FIG. 3. A role for NOX4 component rac1 in Ox-PAPC effects on IL-8 expression

(A) Ox-PAPC causes rac1 recruitment to the cell membrane. HAECs were treated with Ox-PAPC (50ug/ml, Ox50) for the indicated times in M199 containing 1% FBS. The membrane fractions were prepared and the rac1 content in the fractions was determined by Western blotting. The same blot was reprobed with caveolin-1 antibody for normalization. This Western blot was representative of at least 3 repeated experiments with similar results. (B) Rac1 siRNA inhibits IL-8 induction. HAECs were transfected with scrambled (sc) and siRNA to rac1 (rac1 si), and after 2 days of cell growth, the IL-8 induction by Ox-PAPC (50ug/ml, Ox50, 4hr) were measured by QRT-PCR. Values are mean $\pm$ SEMs of triplicate PCR reactions; ** $p < 0.01$.

FIG. 4. Vascular endothelial growth factor receptor 2 (VEGFR2) mediates rac1 recruitment induced by Ox-PAPC in HAEC

(A) HAECs were pretreated with SU1498 (10uM) for 1hr and co-treated with Ox-PAPC (50ug/ml) for an additional 4hrs. The rac1 content in the membrane fractions was determined as in Figure 3A. The relative band densities normalized by caveolin-1 content in the fractions were shown as a graph. (B) HAECs were transfected with scrambled (sc) or VEGFR2-specific siRNA (VEGFR2si) as described in the method section. After 2 days, the relative mRNA levels of VEGFR2 in the cells were determined by QRT-PCR. Values in (B) are mean $\pm$ SEMs of triplicate PCR reactions; *** $p < 0.001$. (C) After 2 days of siRNA transfection, cells were treated with Ox-PAPC (50ug/ml, Ox50) for 4 hrs and the rac1 content in the membrane

fractions were determined as in (A). A representative of at least 3 repeated experiments with similar results is shown.

Table 1
Expression levels of subtypes and components of NOX complex in HAEC and human macrophage.

A. Transcriptional levels of NOX subtype in HAEC									
Gene	GAPDH	NOX1	NOX2	NOX3	NOX4	NOX5	Duox1	Duox2	
Ct	13.90±0.26 5	31.87±0.35 1	35.37±1.87 7	n/d	20.17±0.05 8	31.23±0.60 3	33.90±1.49 3	31.53±0.61 1	
Level*	1000000	3.90	0.35	n/d	12988	6.06	0.95	4.92	
B. Transcriptional levels of regulatory components in HAEC									
Gene	GAPDH	p22 ^{phox}	p47 ^{phox}	p67 ^{phox}	NOXA1	NOXO1	DuoxA1	DuoxA2	
Ct	13.90±0.26 5	18.67±0.35 1	28.33±0.20 8	28.63±0.41 6	25.67±1.05 8	28.10±0.17 3	22.30±0.26 5	n/d	
Level*	1000000	36735	45.2	46.7	287.0	53.1	2960	n/d	
C. Transcriptional levels NOX subtype in human macrophage									
Gene	GAPDH	NOX1	NOX2	NOX3	NOX4	NOX5	Duox1	Duox2	
Ct	14.17±0.15 3	31.90±0.17 3	14.93±0.05 8	n/d	30.80±0.36 1	29.63±0.25 2	30.80±0.62 4	30.90±0.10 0	
Level*	1000000	4.59	587773	n/d	9.84	22.08	9.84	9.18	
D. Transcriptional levels of regulatory components in human macrophage									
Gene	GAPDH	p22 ^{phox}	p47 ^{phox}	p67 ^{phox}	NOXA1	NOXO1	DuoxA1	DuoxA2	
Ct	14.17±0.15 3	14.97±0.15 3	21.00±0.0 0	15.00±0.17 3	22.73±0.20 8	27.93±0.15 3	21.63±0.25 2	n/d	
Level*	1000000	574349	8769	561231	2637	71.8	5653	n/d	

A. Transcriptional levels of NOX subtype in HAEC						
Gene	GAPDH	NOX1	NOX2	NOX3	NOX4	NOX5
					Duox1	Duox2

* Value means mRNA copy number per thousand copies of GAPDH mRNA in HAEC. Copy numbers were calculated from Ct value of QRT-PCR. Each primer set showed ideal double expansion in PCR reactions as determined by standard reactions using known amounts of templates in the reactions. There was no formation of primer dimmers, which was determined by melting dissociation curve. Values are mean $\pm$ SDs of triplicate PCR reactions. n/d: not-detectable.