Original Contribution

**Oxidized-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine induces vascular endothelial superoxide production: Implication of NADPH oxidase**

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**Abstract**

Modified low-density lipoprotein (LDL) induces reactive oxygen species (ROS) production by vascular cells. It is unknown if specific oxidized components in these LDL particles such as oxidized-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (ox-PAPC) can stimulate ROS production. Bovine aortic endothelial cells (BAEC) were incubated with ox-PAPC (50 µg/ml). At 4 h, ox-PAPC significantly enhanced the rate of O$_2^{-}$$/$CO$_2$ production. Pretreatment of BAEC in glucose-free Dulbecco’s modified Eagle’s medium plus 10 mM 2-deoxyglucose (2-DOG), the latter being an antimetabolite that blocks NADPH production by the pentose shunt, significantly reduced the rate of O$_2^{-}$$/$CO$_2$ production. The intensity of NAD(P)H autofluorescence decreased by 28 ± 12% in BAEC incubated with ox-PAPC compared to untreated cells, with a further decrease in the presence of 2-DOG. Ox-PAPC also increased Nox4 mRNA expression by 2.4-fold while pretreatment with apocynin (100 µM) restored the GSH level (control = 22.54 ± 0.23, GSH = 18.06 ± 0.98, apocynin = 22.55 ± 0.60, ox-PAPC + apocynin = 21.17 ± 0.36 nmol/10^6 cells). Treatment with ox-PAPC also increased MMP-2 mRNA expression accompanied by a 1.5-fold increase in MMP-2 activity. Ox-PAPC induced vascular endothelial O$_2^{-}$$/$CO$_2$ production that appears to be mediated largely by NADPH oxidase activity.

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**Keywords:** Ox-PAPC; Superoxide; NAD(P)H oxidase; Matrix metalloproteinases (MMPs)

**Introduction**

Low-density lipoprotein (LDL) oxidation is one of the fundamental processes in atherogenesis. LDL particles trapped within the subendothelial space undergo oxidative modification, resulting in the formation of minimally modified LDL (MM-LDL) and highly oxidized LDL (ox-LDL) particles [1–3]. Oxidized lipids, such as lipid hydroperoxide 13-HPODE, have been implicated in the activation of NAD(P)H oxidase to induce production of O$_2^{-}$$/$ [4]. Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (ox-PAPC), a biologically active component of MM-LDL present in atherosclerotic lesions, is a common product of phosphatidylcholine

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**Abbreviations:** LDL, low-density lipoprotein; MM-LDL, minimally modified LDL; ox-LDL, oxidized LDL; ox-PAPC, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine; ROS, reactive oxygen species; G6PD, glucose-6-phosphate dehydrogenase; eNOS, endothelial nitric oxide synthase; BH$_4$, tetrahydrobiopterin; GPx, glutathione peroxidase; MMPs, matrix metalloproteins; BAEC, bovine aortic endothelial cells; 2-DOG, 2-deoxyglucose; DMEM, Dulbecco’s modified Eagle’s medium; SOD, superoxide dismutase; L-NAME, l-nitroarginine methyl ester; DHE, dihydroethidium; H$_2$DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; SiRNA, small interfering RNA; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DIH, dihydroethidium; RT-PCR, reverse-transcriptase polymerase chain reaction.

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peroxidation in MM-LDL [5–8]. Ox-PAPC is also a potent inducer of monocyte binding to endothelial cells [5,9,10]. However, its ability to stimulate reactive oxygen species (ROS) production has yet to be determined.

The pentose phosphate pathway, which supports the synthesis of riboses for nucleic acid production, is the principal source of NADPH via glucose-6-phosphate dehydrogenase (G6PD). NADPH acts as a cofactor for both NADP+ oxidase and endothelial nitric oxide synthase (eNOS). In addition, NADPH maintains tetrahydrobiopterin (BH4) stores via de novo synthesis. Insufficient arginine or BH4 leads to NOS uncoupling and production of superoxide anion radical (O2−) [11,12]. Reduced NADPH levels reflect an increased oxidative stress state that is, in part, attributable to NADPH oxidase and/or eNOS-mediated ROS production with a concomitant decrease in endothelial-derived NO bioavailability [13,14]. NADPH is also used as a basic source of reducing equivalents to maintain reduced glutathione stores (GSH) that are essential for the scavenging of ROS. ROS are buffered intracellularly by converting GSH to its oxidized form (GSSH) in a reaction catalyzed by glutathione peroxidase (GPx). GSSH is recycled to GSH by glutathione reductase (GSSG reductase), which requires NADPH as a cofactor [15].

The reduced forms NADPH and NADH absorb ultraviolet light at a maximum wavelength of 340 nm. NADPH and NADH are collectively referred to as NAD(P)H [16], and emit a broadband blue fluorescence centered at 450–460 nm while their oxidized forms, namely NADP+ and NADPH, are not fluorescent [17]. In this context, the fluorescence properties of NAD(P)H permit identification and characterization of NADPH levels in the presence of agents that change the cellular redox state [18,19].

Matrix metalloproteins (MMPs) are a family of zinc-dependent enzymes which play a pivotal role in vascular remodeling and destabilization of atherosclerotic plaques [20]. This protease family includes 72-kDa gelatinase A (MMP-2) and 92-kDa gelatinase B (MMP-9) that degrade type IV collagen and gelatin. Shear stress, vascular injury, inflammation, and oxidative stress have been shown to regulate MMP expression and activity via the production of ROS [21–24]. However, it remains unclear whether the ox-PAPC affects vascular endothelial MMP expression and activities.

We demonstrate that ox-PAPC induced vascular endothelial O2− production that appears to be mediated largely by NADPH oxidase activity with accompanied decreases in NADPH autofluorescence. Ox-PAPC induces an oxidant response that seems to modulate MMP-2 activities.

Methods

Experimental protocol

Bovine aortic endothelial cells (BAEC) were cultured to confluent monolayers in standard medium or glucose-free medium with 2-deoxyglucose (2-DOG, 10 mM) (Sigma). BAEC were then treated with ox-PAPC at 50 μg/ml for 4 h. Nox4 expression and matrix metalloproteinase activities were determined at the 4-h interval while O2− formation was measured at various intervals up to 4 h. Samples were visualized for NAD(P)H autofluorescence by fluorescence microscopy. Real-time RT-PCR was performed to quantify the relative levels of Nox4, MMP-2, and MMP-9 mRNA expression in response to ox-PAPC under high glucose vs glucose-free medium with 2-DOG. Western blots were performed for NADPH oxidase subunit, Nox4, protein expression, while MMP activities were measured by zymography.

Endothelial cell culture

BAEC between passages 5 and 9 were seeded on Cell-Tak cell adhesive (Becton Dickson Labware, Bedford, MA) and Vitrogen (Cohesion, Palo Alto, CA; RC 0701) -coated glass Slides (5 cm2) at 3 × 104 cells per slide. BAEC were grown to confluent monolayers in high glucose (4.5 g/L) DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 15% heat-inactivated fetal bovine serum (Hyclone), 100 U/ml penicillin-streptomycin (Irvine Scientific) and 0.05% amphotericin B (GIBCO) for 48 h in 5% CO2 at 37°C.

Ox-PAPC preparation

PAPC (Sigma-Aldrich) was oxidized by transferring 1 mg in 100 μl of chloroform to a clean 16 × 25-mm2 glass test tube and evaporating the solvent under a stream of nitrogen. The lipid residue was allowed to autoxidize while being exposed to air for 24 to 48 h. The extent of oxidation was monitored by electrospay ionization-mass spectrometry in the positive-ion mode [7].

Incubation of BAEC with 2-deoxyglucose and superoxide dismutase (SOD)

To demonstrate whether NADPH, a cofactor, mediates the extent of O2− production, BAEC were incubated with 2-DOG to block the pentose shunt pathway. Once the BAEC monolayers became confluent, growth medium was changed to glucose-free medium comprised of glucose-free DMEM, 15% heat-inactivated FBS, 100 U/ml penicillin-streptomycin, and 0.05% amphotericin B, and 10 mM 2-DOG. No significant differences in cell survival were found under these conditions [25]. Confluent BAEC monolayers were incubated overnight (>12 h), followed by treatment with ox-PAPC at 50 μg/ml for 4 h. Measurement of extracellular O2− formation was performed at 1-, 2-, 3-, and 4-h intervals as described below. In some experiments, cells were pretreated with SOD (60 μg/ml), followed by the addition of ox-PAPC to prevent O2− accumulation and subsequent reactions.
Measurement of extracellular superoxide (O$_2^-$) formation

The production of O$_2^-$ was measured by SOD-inhibited cytochrome c reduction rates as described previously [26,27]. BAEC monolayers on microscope slides were exposed to ox-PAPC for the intervals shown using media containing 100 µM acetylated-ferricytochrome c (Sigma-Aldrich). Control samples were maintained in a cell culture dish with media containing cytochrome c (100 µM) and incubated at 37°C. At set intervals up to 4 h, aliquots of supernatant were taken for absorbance measurements at 550 nm (Beckman DU 640 Spectrophotometer). The specificity of reduction by O$_2^-$ was established by comparing reduction rates in the presence and absence of SOD (60 µg/ml). The corrected rates of O$_2^-$ release were calculated from SOD-inhibitable ferricytochrome c absorbance at 550 nm using the molar extinction coefficient (ε = 21,000 M$^{-1}$ cm$^{-1}$). Extracellular O$_2^-$ production in BAEC in response to ox-PAPC was also measured in the presence of NADPH inhibitor, apocynin (100 µM), xanthine oxidase inhibitor, Allopurinol (100 µM), and mitochondria complex I inhibitor, rotenone (5 µM). In selected experiments, cells were pretreated with L-nitroarginine methyl ester (L-NAME, 100 µM) to inhibit eNOS activity for 60 min before and during incubation with ferricytochrome c to quantify eNOS-dependent O$_2^-$ production [28].

Intracellular O$_2^-$ production measurements

Dihydroethidium (DHE) was used to localize intracellular O$_2^-$ production as previously described [25,27]. Cells are permeable to DHE in the presence of O$_2^-$, and DHE is oxidized to fluorescent ethidium which is trapped by intercalation within the double-stranded nuclear DNA. Confluent BAEC on microscope slides were incubated with 10 µg/ml of DHE (Sigma-Aldrich). After 10 min, cells were washed twice with PBS and then treated with ox-PAPC. Fluorescence microscopy (Olympus) was performed to evaluate the localization of DHE fluorescence (excitation = 518 nm and emission = 605 nm). In parallel, the intracellular redox state was analyzed by 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFH-DA) (Molecular Probes) at 10 µM for 30 min at 37°C. Intracellular oxidant production was also measured using 2',7'-H$_2$DCFH that was oxidized to the fluorescent 2',7'-dichlorofluorescein (excitation = 490 nm and emission = 510 nm) in the presence of hydrogen peroxide and peroxidase [29].

Fluorescence microscopy for NAD(P)H autofluorescence

NAD(P)H autofluorescence from BAEC was imaged using a motorized fluorescence inverted microscope (Carl Zeiss: Axiovert 200, Nomarski DIC, multichannel). The fluorescence emission was selected by a combination of a dichroic mirror (Chroma, 400DCLP) and a bandpass filter (Chroma, DF470 ± 40 nm). Images were recorded and analyzed with a LaVision PicoStar HR12 camera. BAEC were treated with ox-PAPC and/or apocynin as described above. The differential intensities of NAD(P)H autofluorescence were compared from four independent trials. Four arbitrary regions were selected from individual conditions and normalized to the background intensity. The fluorescent intensities from 10 individual BAEC in the field of view of each sample monolayer were averaged and statistically compared with other sets. The fluorescence intensity of NAD(P)H molecules provides a means to image the redox state of intact vascular endothelial cells in response to ox-PAPC and to demonstrates a link among NADPH oxidase activation, O$_2^-$ Production, and NADPH utilization.

Quantitative real-time RT-PCR

After BAEC were exposed to ox-PAPC, total RNA was isolated using RNeasy kit (Qiagen). Real-time RT-PCR was performed according to the recommendations of the PE Biosystems TaqMan PCR Core Reagent Kit [30]. Briefly, equal amounts of RNA at 0.5 µg/µl were reverse-transcribed with rTth DNA polymerase to bring the mixed solution to a final concentration of 1 × TaqMan buffer, 5 mmol/L MgCl$_2$, 200 µmol/L dATP/dCTP/dGTP, 400 µmol/L dUTP, 100 nmol/L probe, 400 nmol/L primers, 0.01 U/µl AmpErase, and 0.025 U/µl AmpliTaq Cold DNA polymerase. Total RNA (0.4 ng/µl in 5 µl) was then transferred to the 96-well plate. PCR were performed at 50°C for 2 min, 60°C for 30 min, and 95°C for 5 min and then run for 60 cycles at 94°C for 20 s and 62°C for 1 min on the real-time RT-PCR Engine (MJ Research Opticon).

$C_t$ is defined as the threshold cycle number at which the initial amplification becomes detectable by fluorescence and the difference in $C_t$ values for various treatment conditions was used to mathematically determine the relative difference in the levels of Nox4, MMP-2, MMP-9, and GAPDH mRNA expression [31]. Relative gene expression ($\Delta$R$_{t}$, was used to normalize fluorescence, assuming that amplification was 100% efficient. TaqMan probes [32] were used for added specificity and sensitivity. The primers (Table 1) were produced on an automated synthesizer (Applied Biosystems) according to the manufacturer’s protocol. For each gene, quantitative RT-PCR was conducted in duplicate.

To ensure the quality of the measurements, both negative and positive controls were systematically included in duplicate in each plate. The statistical analysis of the quantitative RT-PCR results was done using the $\Delta$C$_t$ value ($C_{\text{gene of interest}} - C_{\text{GAPDH}}$). Relative gene expression was obtained by $\Delta$ΔC$_t$ methods ($\Delta$C$_{\text{sample}} - \Delta$C$_{\text{GAPDH}}$) using the control group as a calibrator for comparison of every unknown-sample gene expression level. The conversion between $\Delta$ΔC$_t$ and relative gene expression levels is fold induction = $2^{-\Delta\Delta C_t}$ [33].
Table 1
Primer sequences for bovine Nox4, MMP-2, MMP-9, and GAPDH

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>TaqMan probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox4</td>
<td>5'-AGCAACAAGCGCAGCACATC-3'</td>
<td>5'-FAM-GTCACAAGCATCCGTGAGATGTC-(TAMRA)-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TTCTTTGACCCATCAG-3'</td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>5'-CCCAAGCTCATGCAGGATG-3'</td>
<td>5'-FAM-TGGACGCCCATCCTGATAACCCTGG-(TAMRA)-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGGAAGTCCACACAGCA-3'</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>5'-AGGGAGGCGTCTGCAAC-3'</td>
<td>5'-FAM-ACACCTTTGAGCCATCCTGGG-(TAMRA)-3'</td>
</tr>
<tr>
<td></td>
<td>5'-AGAAATGCAAGGAGCTCTAATC-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CCCACTCCCAACGTCTGTC-3'</td>
<td>5'-VIC-TGTTAGCTGACCTGCGCCTG-(TAMRA)-3'</td>
</tr>
<tr>
<td></td>
<td>5'-ATCTCATCATACTTGGCAGTTTCT-3'</td>
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</tbody>
</table>

Inhibition of Nox4 expression by small interfering RNA (siNox4)

To determine whether NADPH oxidase expression was responsive to ox-PAPC treatment, we inhibited Nox4 expression with small interfering RNA (siRNA). Twenty-one nucleotide siRNA was designed by targeting the Nox4 sequence 5'-AAGACCTGGGCAATATATTAT-3', according to the siRNA design guidelines (www.qiagen.com). Non-silencing fluorescein-labeled control (scrambled siRNA). Double-stranded siRNAs were synthesized (Qiagen-Xeragon) and dissolved in sterile annealing buffer according to the manufacturer’s instruction. The sense mRNA/cDNA target sequences and antisense siRNA sequences were as follows: sense, 5'-GACCCUGGCAGUAAUUUAUd (TT)-3'; antisense, 3'-dT(TT)CUGGACGGGUCUAAUAU-5'. SiRNA transfection to BAEC was monitored with the control siRNA labeled with fluorescein (scrambled siRNA) (Olympus IX70 microscope, Melville, NY). Quantitative RT-PCR were performed to compare Nox4 mRNA degradation in the presence and absence of siNox4 (on-line supplementary).

Measurement of cellular glutathione levels

Intracellular GSH/GSSG levels were measured as previously described [34]. Briefly, cells pellets were deproteinized by using 5% metaphosphoric acid (MPA). The supernatant was isolated and injected to the high-performance liquid chromatography (HPLC) using a reverse-phase C18 column Luna (Phenomenex, Torrance, CA). The mobile phase consisted of sodium phosphate at 25 mM, ion-pairing agent 1-octane sulfonic acid at 0.5 mM, and 2% (v/v) acetonitrile at pH 2.7. The aminothiols were detected by using the CoulArray electrochemical detector (ESA, Inc., Chelmsford, MA).

SDS-PAGE zymography

Gelatinolytic activities in the conditioned media were assayed with gelatin zymography as previously described [35]. Each aliquot of sample was mixed with equal volumes of nonreducing lysis buffer (0.25 M Tris-HCl, pH 6.5; 20% glycerol; 2% SDS, and 10 mg/ml bromophenol blue), after 30 min incubation at room temperature the samples were loaded onto a 10% SDS polyacrylamide gel containing 1 mg/ml of gelatin as a substrate (BioRad Ready Gel, CA). After electrophoresis, the gels were incubated in 2.5% Triton X-100 for 30 min, with two changes of solution, and incubated overnight with the substrate buffer (50 mM Tris, pH 8.0; 50 mM NaCl and 10 mM CaCl2) at 37°C. The gels were then stained with 0.1% Coomassie brilliant blue and destained with 10% acetic acid solution. The gelatinolytic activities were measured by using the NIH Image software program after the gels had been scanned. MMP-2 and MMP-9 were identified based on gelatin lysis at a molecular weight of 72 kDa for MMP-2 and 92 kDa for MMP-9 by a comparison with the MMP-2 and MMP-9 standard (Oncogene, CA).

Statistical analysis

Data are expressed as mean ± SE. Comparison between groups was performed by Student’s paired two-tailed t test. P < 0.05 was considered significant. Significance of differences in optical density of bands from Western and zymography was analyzed by ANOVA and differences between groups were tested with Student’s t test. Differences were taken statistically significant at a value of P < 0.05.

Results

Effect of Ox-PAPC superoxide production

The rates of O₂⁻⁻ production increased steadily in response to ox-PAPC over time (1 h = 0.25 ± 0.02, 2 h = 0.30 ± 0.023, 3 h = 0.34 ± 0.034, and 4 h = 0.37 ± 0.045 nmol/min/million cells) (Fig. 1). The addition of SOD stopped O₂⁻⁻ production over time despite the presence of ox-PAPC (1 h = 0.021 ± 0.02, 2 h = 0.053 ± 0.013, 3 h = 0.065 ± 0.015, 4 h = 0.063 ± 0.019 nmol/min/million cells). BAEC incubated with 2-deoxylucose also revealed a continual decline in O₂⁻⁻ formation over 4 h. Incubating BAEC with both ox-PAPC and 2-DOG resulted in a decrease in the rates of O₂⁻⁻ production. Thus, the presence of SOD or the blockade of the pentose phosphate pathway with 2-DOG resulted in a decrease in O₂⁻⁻ formation.
Ox-PAPC measured by cytochrome c reduction assay. The rate of O$_2^-$ production increased steadily in response to ox-PAPC compared to control. The addition of SOD decreased the rate of O$_2^-$ production by ~2.5-fold. BAEC incubated with 2-deoxyglucose (2-DOG) at 10 mM showed reduced O$_2^-$ production over 4 h compared to control. The addition of ox-PAPC at 50 μg/ml to BAEC incubated with 2-DOG induced a rate of O$_2^-$ production comparable to that of control, and then decreased at 4 h. Ox-PAPC minus control vs. Ox-PAPC + DOG minus DOG was also statistically significant (P < 0.05, n = 5). The specificity for reduction by O$_2^-$ was established by parallel measurements in the presence of SOD (60 μg/ml).

To visualize the effect of ox-PAPC on intracellular O$_2^-$ formation, we incubated BAEC with dihydroethidium (DHE). The merged images of phase and fluorescence microscopy at 4 h demonstrate the localization of red fluorescence in nuclei (Fig. 2b). Increased formation of O$_2^-$ is evident from an increase in ethidium red fluorescence after ox-PAPC treatment (Fig. 2c). In parallel, ox-PAPC-treated BAEC showed an increase in 2,7-DCF fluorescent intensities over time (Fig. 2d). The formation of ethidium red fluorescence was inhibited in the presence of SOD and PEG-SOD.

**The effects of Ox-PAPC on NADPH oxidase subunit, Nox4**

The increased endothelial O$_2^-$ formation was coupled with Nox4 mRNA expression. Ox-PAPC significantly up-regulated Nox4 mRNA expression (Fig. 3). When BAEC were transfected with Nox4-specific siRNA and a non-silencing siRNA (scrambled siRNA) as a control, Nox4 siRNA significantly reduced mRNA levels by 80.8 ± 2.45% at 4 h whereas Nox4 mRNA following treatment with scrambled siNox4 remained unchanged. SiNox4 also decreased ox-PAPC-mediated up-regulation of Nox4 mRNA expression, suggesting that the increase in O$_2^-$ formation induced by ox-PAPC may be mediated by Nox4.

Addition of the NADPH oxidase inhibitor, apocynin, reduced the rate of O$_2^-$ production by 3.25-fold ± 0.03 in ox-PAPC-treated BAEC, whereas additions of allopurinol or rotenone reduced O$_2^-$ production rates by 0.40-fold ± 0.05, and 0.31-fold ± 0.04, respectively (Fig. 4). Furthermore, addition of ox-PAPC to L-NAME-pretreated BAEC increased the production of O$_2^-$ in comparison with the addition of ox-PAPC alone. These observations suggest that ox-PAPC induced O$_2^-$ production largely by up-regulating NADPH oxidase, and that this correlated with increased extra- and intracellular production of ROS.

We next measured cellular GSH levels in the presence of ox-PAPC and apocynin to determine if GSH affects the redox state of BAEC. Treatment with ox-PAPC depleted the GSH store while pretreatment with apocynin restored the GSH level (control = 22.54 ± 0.23, ox-PAPC = 18.06 ± 0.98, apocynin = 22.54 ± 0.60, apocynin + ox-PAPC = 21.17 ± 0.36 nmol/million cells, n = 4, P < 0.001) (Fig. 5). The level of GSH in cells pretreated with apocynin, followed by ox-PAPC, was similar to that of the control and apocynin alone with no detectable GSSG level (n = 4, P = NS). The levels of oxidized GSH (GSSG) were beyond the detection limit in the control and apocynin samples except for the ox-PAPC-treated sample (ox-PAPC: GSSG = 0.49 ± 0.33 nmol/million cells).

**NAD(P)H autofluorescence**

The intensity of NAD(P)H autofluorescence was analyzed in control samples (without treatment), in samples incubated with ox-PAPC, an in samples treated in the presence of 2-DOG, as well as with both ox-PAPC and 2-DOG. BAEC incubated with ox-PAPC revealed a statistically significant decrease in fluorescent intensity by 28 ± 12% compared to controls, suggesting that activation of NADPH oxidase, concurrent production of O$_2^-$, and possible increases in cell oxidative stress accounted for depletion of the NADPH pool (Fig. 6). NADP+ and NAD+ do not behave as fluorophores [17]. Blockade of the pentose phosphate shunt with 2-DOG resulted in a 55.6 ± 26% decrease in NAD(P)H autofluorescence compared to control cells. Similarly, a decrease in NAD(P)H fluorescence intensity was noted in samples treated with both 2-DOG and ox-PAPC (22.3 ± 7.2%, P < 0.05, n = 4). Notably, the extent of this decrease, compared to 2-DOG alone, was not statistically significant, suggesting that both deplete the same pool. NADPH oxidase inhibition by apocynin partially restored the NAD(P)H autofluorescence in the Ox-PAPC-treated BAEC, further suggesting that the NADPH oxidase is activated by ox-PAPC.

**Ox-PAPC and MMP-2 and -9**

To further assess the physiological importance of O$_2^-$ generated by BAEC exposed to ox-PAPC, we
tested MMP expression on the premise that MMP activity is modulated by ROS [20]. In the presence of ox-PAPC, the MMP-2 mRNA was up-regulated whereas MMP-9 was down-regulated compared with the control \( (n = 4, P < 0.05) \) (Fig. 7a). To verify that the observed effects were mediated by \( \text{O}_2^- \)/\( \text{CO}_2 \), we showed that SOD attenuated the MMP-2 activities by 25 \( \% \), whereas ox-PAPC increased MMP-2 activity by 65 \( \% \) \( \pm \) 29 \( \% \) (Figs. 7b and 7c). The combination of SOD and ox-PAPC decreased MMP-2 activity by 30 \( \% \) \( \pm \) 23 \( \% \) \( (P < 0.05, n = 3) \).

**Discussion**

Our findings show that ox-PAPC treatment of BAEC induces a generation of \( \text{O}_2^- \). This effect appears to be mediated largely by induction of NADPH oxidase activity and Nox4 expression. Increased \( \text{O}_2^- \) production in vascular endothelium likely occurs through a succession of events that include rapid assembly and activation of existing NADPH oxidase components [36], followed by a prolonged increase in \( \text{O}_2^- \) production through the further expression of Nox4 [37,38]. The production of ROS may be part of a complex...
series of biochemical events that recruit monocytes binding to the endothelium. The generation of ROS in the environment surrounding endothelial cells exposed to oxidants, or to products of oxidation, may be a mechanism by which an inflammatory response is induced.

Generation of O$_2^•^-$/H$_2$O$_2$ and subsequent dismutation to H$_2$O$_2$ are expected to increase glutathione peroxidase activity and a demand on the GSH pool[39]. After treatment with ox-PAPC, a decrease in GSH/GSSG ratios was observed. The intensity of NAD(P)H autofluorescence decreased while the rates of O$_2^•^-$/H$_2$O$_2$ production increased. A similar and non-additive decrease in GSH was observed by inhibition of the pentose shunt with 2-DOG, providing evidence that ox-PAPC reduces NADPH. Depletion of a NADPH pool could account for O$_2^•^-$/H$_2$O$_2$ production, since regeneration of GSH from GSSG via GSH reductase could account for the self-limiting endothelial production of O$_2^•^-$/H$_2$O$_2$ by NADPH oxidase. NAD(P)H autofluorescence intensity in ox-PAPC-treated BAEC provides a noninvasive method to show that stimulated NADPH oxidase activity decreases the cellular reserve of reducing equivalents in the form of NADH and NADPH in cells. Leopold et al. demonstrated that overexpression of G6PD, which enhances NADPH production by the pentose phosphate pathway, decreases ROS accumulation[15]. In this context, the intensity of NAD(P)H autofluorescence may represent an index of cellular oxidative stress or of the demand for reducing equivalents.
in response to an oxidative challenge. In the presence of ox-PAPC, NAD(P)H autofluorescence was attenuated in parallel with an increase in Nox4 expression. The rates of O$_2^•$ production declined after 5 h (data not shown), possibly reflecting a depletion of NADPH pools.

ROS have been previously implicated in the regulation of MMPs [40]. SOD was used to assess whether O$_2^•$ production mediates the effects of ox-PAPC (Fig. 7c). Our findings suggest that part of this modulation may take place through an oxidative process initiated by ox-PAPC. However, the precise mechanism(s) by which ox-PAPC induces MMP activities remain to be defined.

Potential vascular sources of O$_2^•$ include xanthine oxidase (XO), cyclooxygenase, uncoupling of NO synthases (NOS) [11,41–44], and NAD(P)H oxidases (multisubunit membrane complexes) [42], as well as the mitochondrial respiratory chain [45] and hemoglobin [46,47], the relative importance of which may vary among vascular beds, types of cells, and disease conditions. Recent studies suggest that in coronary arteries, NAD(P)H oxidases of the NOX family are a predominant source of O$_2^•$ from noninflammatory cells such as endothelium [48,49]. The formation of O$_2^•$ by the endothelial NADPH oxidase accounts for the reduced NO bioavailability, development of endothelial dysfunction from the aortic ring of gp91phox-knockout mice (gp91phox-/-) [50]. In this context, apocynin (4-hydroxy-3-methoxy-acetophenone) has been demonstrate to act as a potent and selective inhibitor of NADPH oxidase system in both in vitro and animal models [51–53] by interfering with the translocation of an essential cytosolic protein, p47phox [54–56]. Diphenylene iodonium (DPI) nonspecifically inhibits flavoprotein-containing enzymes, including NADPH oxidases, NOS, and complex I of mitochondrial electron transport chain [57]. Allopurinol has been reported to inhibit xanthine oxidase-derived oxygen free radical production [58]. Also, rotenone inhibits mitochondrial respiratory chain complex I, specifically, electron transfer within the NADH-Q reductase complex [59].

Several O$_2^•$-forming enzyme systems have been identified in vascular cells that utilize NADPH or NADH as a cofactor. These include vascular homologues of the leukocyte NADPH oxidase [38], cytochrome P450 [60], the mitochondrial electron transport chain [61], and uncoupled NO synthase [62]. Ox-PAPC may impair endothelial cell function by uncoupling endothelial nitric oxide synthase activity in a manner analogous to the effects of LDL. Impairment of eNOS could allow O$_2^•$ to be generated either directly from uncoupled eNOS or from decreased NO production that would otherwise react with O$_2^•$ [28]. Our findings indicate that in endothelial cells exposed to ox-PAPC, most O$_2^•$ formation is derived from the NAD(P)H oxidase, with minor amounts of O$_2^•$ possibly including xanthine oxidase and the mitochondrial electron transport chain.

Confluent BAEC were pretreated with 2-DOG overnight (>12 h) prior to the addition of ox-PAPC. BAEC remained viable compared to the control as reported previously [25]. 2-DOG has been widely used to study intracellular redox status in pulmonary arterial endothelial cell electron transport [63,64]. Although 2-DOG was not a specific NADPH inhibitor, others such as 6-amino-nicotinamide [65] to inhibit NADPH regeneration via the pentose phosphate pathway can also be toxic and nonspecific.

Nox1, Nox2 (gp91phox), and Nox4 may account for O$_2^•$ production [38]. Rueckschloss et al. showed that ox-LDL induces proatherosclerotic NADPH oxidase subunit, gp91phox, expression and O$_2^•$ formation in human endothelial cells [37]. Both Nox1 and Nox2 were reported for NADPH oxidase-mediated O$_2^•$ in vascular smooth muscle cells [66,67]. Nox4 is considered the most abundant Nox enzyme in vascular endothelial cells [38]. We have
previously shown that Nox4 is more abundantly expressed than Nox2 in cultured BAEC in response to fluid shear stress [27]. In static cultured endothelial cells obtained from the thoracic aortas of C57BL/6, Nox4 mRNA was reported to be far more abundant than Nox1 and Nox2 [68]. To show intracellular O$_2$$^-$ production, we used dihydroethidium and 2',7'-dichlorodihydrofluorescein diacetate to account for Nox4 activity in BAEC [69]. To show extracellular O$_2$$^-$ formation, we used cytochrome c assay to account for the activities of Nox1 and Nox2 [27].

Although the specific mechanism(s) by which ox-PAPC induces NADPH oxidase activity and O$_2$$^-$ production remain to be defined, the effect may be similar to that reported for lysophosphatidylcholine (LPS) which stimulates monocyte chemoattractant protein-1 (MCP-1) expression and O$_2$$^-$ production by NADPH oxidase. Similar activation is also reported for other lysolipids such as phosphatidic acid and platelet activating factor, which induce mitogenic signaling pathways. Notably, a substantial increase in NADPH-stimulated O$_2$$^-$ formation was reported in coronary arteries from patients with coronary artery disease [70]. Thus, among other ox-LDL-associated components that stimulate vascular inflammatory responses, ox-PAPC represents a species that can promote the signaling of ROS production.

Conclusion

Minimally modified LDL species containing ox-PAC induced vascular endothelial cells to generate O$_2$$^-$ production largely via endothelial NADPH oxidase with accompanied decreases in NAD(P)H autofluorescence. These findings suggest that ox-PAPC stimulates a proinflammatory response in vascular cells that largely utilizes NADPH oxidase as a source of ROS to mediate redox-sensitive signaling processes.

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