Published in final edited form as:

Free Radic Biol Med. 2005 December 1; 39(11): 1512–1522. doi:10.1016/j.freeradbiomed.2005.07.013.

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

Mahsa Rouhanizadeh^a, Juliana Hwang^b, Roza E. Clempus^c, Laura Marcu^a, Bernard Lassègue^c, Alex Sevanian^{b,†}, and Tzung K. Hsiai^{a,b,*}

^a Department of Biomedical Engineering and Division of Cardiovascular Medicine, University of Southern California, Los Angeles, CA 90089-1451, USA

^b Department of Molecular Pharmacology & Toxicology and the Atherosclerosis Research Unit, University of Southern California, Los Angeles, CA, USA

^c Division of Cardiology, Emory University, Atlanta, GA, USA

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 O₂^{•−} 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 O₂^{•−} 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 ± 0.1 while pretreatment of BAEC with the small interfering RNA (siNox4) attenuated Nox4 RNA expression. Ox-PAPC further reduced the level of glutathione 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/10⁶ 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 O₂^{•−} production that appears to be mediated largely by NADPH oxidase activity.

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

* Corresponding author. E-mail address: E-mail: hsiai@usc.edu (T.K. Hsiai)..

† Deceased.

implicated in the activation of NAD(P)H oxidase to induce production of $O_2^{\bullet-}$ [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 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 NADPH oxidase and endothelial nitric oxide synthase (eNOS). In addition, NADPH maintains tetrahydrobiopterin (BH_4) stores via de novo synthesis. Insufficient arginine or BH_4 leads to NOS uncoupling and production of superoxide anion radical ($O_2^{\bullet-}$) [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 NAD^+ and $NADP^+$, 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 $O_2^{\bullet-}$ production that appears to be mediated largely by NADPH oxidase activity with accompanied decreases in NAD(P)H 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 μ g/ml for 4 h. Nox4 expression and matrix metalloproteinase activities were determined at the 4-h interval while $O_2^{\bullet-}$ 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 3×10^6 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 autooxidize while being exposed to air for 24 to 48 h. The extent of oxidation was monitored by electrospray 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 (O₂^{•-}) formation

The production of O₂^{•-} 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* (Sigmund-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 O₂^{•-} was established by comparing reduction rates in the presence and absence of SOD (60 µg/ml). The corrected rates of O₂^{•-} release were calculated from SOD-inhibitable ferricytochrome *c* absorbance at 550 nm using the molar extinction coefficient ($\epsilon = 21,000 \text{ M}^{-1} \text{ cm}^{-1}$). Extracellular O₂^{•-} 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 O₂^{•-} production [28].

Intracellular O₂^{•-} production measurements

Dihydroethidium (DHE) was used to localize intracellular O₂^{•-} production as previously described [25,27]. Cells are permeable to DHE in the presence of O₂^{•-}, 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 (H₂DCFH-DA) (Molecular Probes) at 10 μ M for 30 min at 37°C. Intracellular oxidant production was also measured using 2',7'-H₂DCFH that was oxidized to the fluorescent 2',7'-dichlorofluorescein (excitation = 490 nm and emission = 610 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 $\pm$ 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 demonstrate a link among NADPH oxidase activation, O₂^{-•} 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 μ g/ μ L were reverse-transcribed with rTth DNA polymerase to bring the mixed solution to a final concentration of 1 $\times$ TaqMan buffer, 5 mmol/L MgCl₂, 200 μ mol/L dATP/dCTP/dGTP, 400 μ mol/L dUTP, 100 nmol/L probe, 400 nmol/L primers, 0.01 U/ μ L AmpErase, and 0.025 U/ μ L AmpliTag Cold DNA polymerase. Total RNA (0.4 ng/ μ L in 5 μ L) 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 (ΔR_n), 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 ΔC_t value ($C_{t\text{gene of interest}} - \Delta C_{t\text{GAPDH}}$). Relative gene expression was obtained by $\Delta\Delta C_t$ methods ($\Delta C_{t\text{sample}} - \Delta C_{t\text{GAPDH}}$) using the control group as a calibrator for comparison of every unknown-sample gene expression level. The conversion between $\Delta\Delta C_t$ and relative gene expression levels is fold induction = $2^{-\Delta\Delta C_t}$ [33].

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'-AAGACCTGGCCAGTATATTAT-3', according to the siRNA design guidelines

(www.qiagen.com). Non-silencing fluorescein-labeled control (5 nM) was used as the negative siRNA 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'-GACCUGGCCAGUAUAUUAUd (TT)-3'; antisense, 3'-d(TT)CUGGACCGGUCAUAUAAUA-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 $\pm$ 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 $\pm$ 0.02, 2 h = 0.30 $\pm$ 0.023, 3 h = 0.34 $\pm$ 0.034, and 4 h = 0.37 $\pm$ 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 $\pm$ 0.02, 2 h = 0.053 $\pm$ 0.013, 3 h = 0.065 $\pm$ 0.015, 4 h = 0.063 $\pm$ 0.019 nmol/min/million cells). BAEC incubated with 2-deoxyglucose 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 $O_2^{\bullet-}$ production. Thus, the presence of SOD or the blockade of the pentose phosphate pathway with 2-DOG resulted in a decrease in $O_2^{\bullet-}$ formation.

To visualize the effect of ox-PAPC on intracellular $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $80.8 \pm 2.45\%$ 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 $O_2^{\bullet-}$ formation induced by ox-PAPC may be mediated by Nox4.

Addition of the NADPH oxidase inhibitor, apocynin, reduced the rate of $O_2^{\bullet-}$ production by 3.25-fold ± 0.03 in ox-PAPC-treated BAEC, whereas additions of allopurinol or rotenone reduced $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ in comparison with the addition of ox-PAPC alone. These observations suggest that ox-PAPC induced $O_2^{\bullet-}$ 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 ($n = 4$, $P = \text{NS}$). 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, 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 $28 \pm 12\%$ compared to controls, suggesting that activation of NADPH oxidase, concurrent production of $O_2^{\bullet-}$, 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 $55.6 \pm 26\%$ 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 ($22.3 \pm 7.2\%$, $P < 0.05$, $n = 4$). 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$, we showed that SOD attenuated the MMP-2 activities by $25 \pm 10\%$, 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 $O_2^{\bullet-}$. This effect appears to be mediated largely by induction of NADPH oxidase activity and Nox4 expression. Increased $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ and subsequent dismutation to H_2O_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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production, since regeneration of GSH from GSSG via GSH reductase could account for the self-limiting endothelial production of $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ from noninflammatory cells such as endothelium [48,49]. The formation of $O_2^{\bullet-}$ 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 demonstrated 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 $O_2^{\bullet-}$ -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 $O_2^{\bullet-}$ to be generated either directly from uncoupled eNOS or from decreased NO production that would otherwise react with $O_2^{\bullet-}$ [28]. Our findings indicate that in endothelial cells exposed to ox-PAPC, most $O_2^{\bullet-}$ formation is derived from the NAD(P)H oxidase, with minor amounts of $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production [38]. Rueckschloss et al. showed that ox-LDL induces proatherosclerotic NADPH oxidase subunit, gp91phox, expression and $O_2^{\bullet-}$ formation in human endothelial cells [37]. Both Nox1 and Nox2 were reported for NADPH oxidase-mediated $O_2^{\bullet-}$ 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 [25]. In static cultured endothelial cells obtained from the thoracic aortas of C57BL6, Nox4 mRNA was reported to be far more abundant than Nox1 and Nox2 [68]. To show intracellular $O_2^{\bullet-}$ production, we used dihydroethidium and 2',7'-dichlorodihydrofluorescein diacetate to account for Nox4 activity in BAEC [69]. To show extracellular $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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

O₂^{•-} 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 O₂^{•-} 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.

Acknowledgements

This paper is dedicated to Dr. Alex Sevanian who passed away in the beginning of 2005. The authors appreciate the bovine Nox4 antibody generously provided by J. David Lambeth of Emory University School of Medicine, Pathology and Laboratory Medicine. T.K.H. is supported by Grants AHA BGIA (0265166U), NIH (K08 HL068689-01A1), and National Heart Foundation/American Health Assistance Foundation (AHAF: H2003-028). L.M. is supported by NIH Grant R01 HL67377.

References

- Berliner JA, Heinecke JW. The role of oxidized lipoproteins in atherogenesis. *Free Radic Biol Med* 1996;20:707–727. [PubMed: 8721615]
- Witztum JR. The oxidation hypothesis of atherosclerosis. *Lancet* 1994;344:793–795. [PubMed: 7916078]
- Steinberg D, Witztum JL. Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? *Circulation* 2002;105:2107–2111. [PubMed: 11980692]
- Li WG, Stoll LL, Rice JB, Xu SP, Miller FJ Jr, Chatterjee P, Hu L, Oberley LW, Spector AA, Weintraub NL. Activation of NAD(P)H oxidase by lipid hydroperoxides: mechanism of oxidant-mediated smooth muscle cytotoxicity. *Free Radic Biol Med* 2003;34:937–946. [PubMed: 12654483]
- Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci USA* 1990;87:5134–5138. [PubMed: 1695010]
- Navab M, Berliner JA, Watson AD, Hama SY, Territo MC, Lusis AJ, Shih DM, Van Lenten BJ, Frank JS, Demer LL, Edwards PA, Fogelman AM. The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. *Arterioscler Thromb Vasc Biol* 1996;16:831–842. [PubMed: 8673557]
- Watson AD, Leitinger N, Navab M, Faull KF, Horkko S, Witztum JL, Palinski W, Schwenke D, Salomon RG, Sha W, Subbanagounder G, Fogelman AM, Berliner JA. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. *J Biol Chem* 1997;272:13597–13607. [PubMed: 9153208]
- Reddy ST, Grijalva V, Ng C, Hassan K, Hama S, Mottahedeh R, Wadleigh DJ, Navab M, Fogelman AM. Identification of genes induced by oxidized phospholipids in human aortic endothelial cells. *Vasc Pharmacol* 2002;38:211–218.
- Navab M, Fogelman AM, Berliner JA, Territo MC, Demer LL, Frank JS, Watson AD, Edwards PA, Lusis AJ. Pathogenesis of atherosclerosis. *Am J Cardiol* 1995;76:18C–23C.
- Hsiai T, Cho SK, Honda H, Hama S, Reddy S, Navab M, Demer L, Ho CM. Pulsatile flow patterns modulate monocyte adhesion to endothelial cells in response to oxidized lipid. *Arterioscler Thromb Vasc Biol*. 2004

11. Xia Y, Tsai AL, Berka V, Zweier JL. Superoxide generation from endothelial nitric-oxide synthase. A Ca²⁺/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J Biol Chem* 1998;273:25804–25808. [PubMed: 9748253]
12. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA Jr. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci USA* 1998;95:9220–9225. [PubMed: 9689061]
13. Leopold JA, Cap A, Scribner AW, Stanton RC, Loscalzo J. Glucose-6-phosphate dehydrogenase deficiency promotes endothelial oxidant stress and decreases endothelial nitric oxide bioavailability. *FASEB J* 2001;15:1771–1773. [PubMed: 11481225]
14. Mueller CF, Laude K, McNally JS, Harrison DG. Redox mechanisms in blood vessels. *Arterioscler Thromb Vasc Biol* 2005;25:274–278. [PubMed: 15514203]
15. Leopold JA, Zhang YY, Scribner AW, Stanton RC, Loscalzo J. Glucose-6-phosphate dehydrogenase overexpression decreases endothelial cell oxidant stress and increases bioavailable nitric oxide. *Arterioscler Thromb Vasc Biol* 2003;23:411–417. [PubMed: 12615686]
16. Stephens DJ, Allan VJ. Light microscopy techniques for live cell imaging. *Science* 2003;300:82–86. [PubMed: 12677057]
17. Huang S, Heikal AA, Webb WW. Two-photon fluorescence spectroscopy and microscopy of NAD (P)H and flavoprotein. *Biophys J* 2002;82:2811–2825. [PubMed: 11964266]
18. Piston DW, Knobel SM. Quantitative imaging of metabolism by two-photon excitation microscopy. *Methods Enzymol* 1999;307:351–368. [PubMed: 10506984]
19. Dellinger M, Geze M, Santus R, Kohen E, Kohen C, Hirschberg JG, Monti M. Imaging of cells by autofluorescence: a new tool in the probing of biopharmaceutical effects at the intracellular level. *Biotechnol Appl Biochem* 1998;28(Pt 1):25–32. [PubMed: 9693085]
20. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest* 1996;98:2572–2579. [PubMed: 8958220]
21. Galis ZS, Asanuma K, Godin D, Meng X. N-acetyl-cysteine decreases the matrix-degrading capacity of macrophage-derived foam cells: new target for antioxidant therapy? *Circulation* 1998;97:2445–2453. [PubMed: 9641697]
22. Fukai T, Galis ZS, Meng XP, Parthasarathy S, Harrison DG. Vascular expression of extracellular superoxide dismutase in atherosclerosis. *J Clin Invest* 1998;101:2101–2111. [PubMed: 9593766]
23. Magid R, Murphy TJ, Galis ZS. Expression of matrix metalloproteinase-9 in endothelial cells is differentially regulated by shear stress. Role of c-Myc. *J Biol Chem* 2003;278:32994–32999. [PubMed: 12816956]
24. Cipollone F, Iezzi A, Fazia M, Zucchelli M, Pini B, Cuccurullo C, De Cesare D, De Blasis G, Muraro R, Bei R, Chiarelli F, Schmidt AM, Cuccurullo F, Mezzetti A. The receptor RAGE as a progression factor amplifying arachidonate-dependent inflammatory and proteolytic response in human atherosclerotic plaques: role of glycemic control. *Circulation* 2003;108:1070–1077. [PubMed: 12912808]
25. Hwang J, Ing MH, Salazar A, Lassegue B, Griendling KK, Navab M, Sevanian A, Hsiai TK. Pulsatile vs. oscillatory shear stress regulates NADPH oxidase system: implication for native LDL oxidation. *Circ Res* 2003;93:1225–1232. [PubMed: 14593003]
26. Hwang J, Wang J, Morazzoni P, Hodis HN, Sevanian A. The phytoestrogen equol increases nitric oxide availability by inhibiting superoxide production: an antioxidant mechanism for cell-mediated LDL modification. *Free Radic Biol Med* 2003;34:1271–1282. [PubMed: 12726915]
27. De Keulenaer GW, Chappell DC, Ishizaka N, Nerem RM, Alexander RW, Griendling KK. Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase. *Circ Res* 1998;82:1094–1101. [PubMed: 9622162]
28. Pritchard KA Jr, Groszek L, Smalley DM, Sessa WC, Wu M, Villalon P, Wolin MS, Stemerman MB. Low density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. *Circ Res* 1995;77:510–518. [PubMed: 7543827]
29. Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic Biol Med* 1999;27:612–616. [PubMed: 10490282]

30. Chu Y, Heistad DD, Knudtson KL, Lamping KG, Faraci FM. Quantification of mRNA for endothelial NO synthase in mouse blood vessels by real-time polymerase chain reaction. *Arterioscler Thromb Vasc Biol* 2002;22:611–616. [PubMed: 11950699]
31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-delta-deltaCT method. *Methods* 2001;25(4):402–408. [PubMed: 11846609]
32. Walker NJ. A technique whose time has come. *Science* 2002;296:557–559. [PubMed: 11964485]
33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-(delta)(delta)Ct method. *Methods* 2001;25:402–408. [PubMed: 11846609]
34. Rebrin I, Kamzalov S, Sohal RS. Effects of age and caloric restriction on glutathione redox state in mice. *Free Radic Biol Med* 2003;35:626–635. [PubMed: 12957655]
35. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Unemori EN, Lark MW, Amento E, Libby P. Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. *Circ Res* 1994;75:181–189. [PubMed: 8013077]
36. Ambasta RK, Kumar P, Griendling KK, Schmidt HH, Busse R, Brandes RP. Direct interaction of the novel Nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase. *J Biol Chem* 2004;279:45935–45941. [PubMed: 15322091]
37. Rueckschloss U, Galle J, Holtz J, Zerkowski HR, Morawietz H. Induction of NAD(P)H oxidase by oxidized low-density lipoprotein in human endothelial cells: antioxidative potential of hydroxymethylglutaryl coenzyme A reductase inhibitor therapy. *Circulation* 2001;104:1767–1772. [PubMed: 11591612]
38. Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK. Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation* 2002;105:1429–1435. [PubMed: 11914250]
39. Hojo Y, Saito Y, Tanimoto T, Hoefen RJ, Baines CP, Yamamoto K, Haendeler J, Asmis R, Berk BC. Fluid shear stress attenuates hydrogen peroxide-induced c-Jun NH2-terminal kinase activation via a glutathione reductase-mediated mechanism. *Circ Res* 2002;91:712–718. [PubMed: 12386148]
40. Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 1994;94:2493–2503. [PubMed: 7989608]
41. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res* 2002;91:406–413. [PubMed: 12215489]
42. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 2000;86:494–501. [PubMed: 10720409]
43. Landmesser U, Harrison DG. Oxidant stress as a marker for cardiovascular events: Ox marks the spot. *Circulation* 2001;104:2638–2640. [PubMed: 11723010]
44. Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. *J Biol Chem* 2003;278:22546–22554. [PubMed: 12692136]
45. Ide T, Tsutsui H, Kinugawa S, Utsumi H, Kang D, Hattori N, Uchida K, Arimura K, Egashira K, Takeshita A. Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. *Circ Res* 1999;85:357–363. [PubMed: 10455064]
46. Balagopalakrishna C, Manoharan PT, Abugo OO, Rifkind JM. Production of superoxide from hemoglobin-bound oxygen under hypoxic conditions. *Biochemistry* 1996;35:6393–6398. [PubMed: 8639585]
47. Mirsa H, Fridovitch I. The generation of superoxide radical during the autooxidation of hemoglobin. *J Biol Chem* 1972;247:6960–6962. [PubMed: 4673289]
48. Mohazzab KM, Kaminski PM, Wolin MS. NADH oxido-reductase is a major source of superoxide anion in bovine coronary artery endothelium. *Am J Physiol* 1994;266:H2568–H2572. [PubMed: 8024019]
49. Liu JQ, Zelko IN, Folz RJ. Reoxygenation-induced constriction in murine coronary arteries: the role of endothelial NADPH oxidase (gp91phox) and intracellular superoxide. *J Biol Chem* 2004;279:24493–24497. [PubMed: 15070892]

50. Jung O, Schreiber JG, Geiger H, Pedrazzini T, Busse R, Brandes RP. gp91phox-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension. *Circulation* 2004;109:1798–1801.
51. Dodd OJ, Pearse DB. Effect of the NADPH oxidase inhibitor apocynin on ischemia-reperfusion lung injury. *Am J Physiol Heart Circ Physiol* 2000;279:H303–H312. [PubMed: 10899070]
52. Dong F, Zhang X, Wold LE, Ren Q, Zhang Z, Ren J. Endothelin-1 enhances oxidative stress, cell proliferation and reduces apoptosis in human umbilical vein endothelial cells: role of ETB receptor, NADPH oxidase and caveolin-1. *Br J Pharmacol* 2005;145:323–333. [PubMed: 15765100]
53. Peng T, Lu X, Feng Q. Pivotal role of gp91phox-containing NADH oxidase in lipopolysaccharide-induced tumor necrosis factor- α expression and myocardial depression. *Circulation* 2005;111:1637–1644. [PubMed: 15795323]
54. van de Worm EFR, DeLeo CJ, Beukelman AJJ, van den Berg RP, Labadie HvD, Nauseef WM. Apocynin, a inhibitor of NADPH oxidase-dependent ROS production in stimulated human neutrophils. 1997
55. Hamilton CA, Brosnan MJ, Al-Benna S, Berg G, Dominiczak AF. NAD(P)H oxidase inhibition improves endothelial function in rat and human blood vessels. *Hypertension* 2002;40:755–762. [PubMed: 12411473]
56. Oeckler RA, Kaminski PM, Wolin MS. Stretch enhances contraction of bovine coronary arteries via an NAD(P)H oxidase-mediated activation of the extracellular signal-regulated kinase mitogen-activated protein kinase cascade. *Circ Res* 2003;92:23–31. [PubMed: 12522117]
57. Holland PC, Clark MG, Bloxham DP, Lardy HA. Mechanism of action of the hypoglycemic agent diphenyleneiodonium. *J Biol Chem* 1973;248:6050–6056. [PubMed: 4726296]
58. Doehner W, Schoene N, Rauchhaus M, Leyva-Leon F, Pavitt DV, Reaveley DA, Schuler G, Coats AJ, Anker SD, Hambrecht R. Effects of xanthine oxidase inhibition with allopurinol on endothelial function and peripheral blood flow in hyperuricemic patients with chronic heart failure: results from 2 placebo-controlled studies. *Circulation* 2002;105:2619–2624. [PubMed: 12045167]
59. Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. *Arterioscler Thromb Vasc Biol* 2005;25:29–38. [PubMed: 15539615]
60. Fleming I. Cytochrome p450 and vascular homeostasis. *Circ Res* 2001;89:753–762. [PubMed: 11679404]
61. Waypa GB, Marks JD, Mack MM, Boriboun C, Mungai PT, Schumacker PT. Mitochondrial reactive oxygen species trigger calcium increases during hypoxia in pulmonary arterial myocytes. *Circ Res* 2002;91:719–726. [PubMed: 12386149]
62. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG. Oxidation of tetra-hydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 2003;111:1201–1209. [PubMed: 12697739]
63. Merker MP, Bongard RD, Kettenhofen NJ, Okamoto Y, Dawson CA. Intracellular redox status affects transplasma membrane electron transport in pulmonary arterial endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L36–L43. [PubMed: 11741813]
64. Leach RM, Hill HM, Snetkov VA, Robertson TP, Ward JP. Divergent roles of glycolysis and the mitochondrial electron transport chain in hypoxic pulmonary vasoconstriction of the rat: identity of the hypoxic sensor. *J Physiol* 2001;536:211–224. [PubMed: 11579170]
65. Paravicini TM, Gulluyan LM, Dusting GJ, Drummond GR. Increased NADPH oxidase activity, gp91phox expression, and endothelium-dependent vasorelaxation during neointima formation in rabbits. *Circ Res* 2002;91:54–61. [PubMed: 12114322]
66. Touyz RM, Chen X, Tabet F, Yao G, He G, Quinn MT, Pagano PJ, Schiffrin EL. Expression of a functionally active gp91phox-containing neutrophil-type NAD(P)H oxidase in smooth muscle cells from human resistance arteries: regulation by angiotensin II. *Circ Res* 2002;90(11):1205–1213. [PubMed: 12065324]
67. Lassegue B, Sorescu D, Szocs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK. Novel gp91(phox) homologues in vascular smooth muscle cells: nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ Res* 2001;88:888–894. [PubMed: 11348997]

68. Sorescu GP, Song H, Tressel SL, Hwang J, Dikalov S, Smith DA, Boyd NL, Platt MO, Lassegue B, Griendling KK, Hanjoong J. Bone morphogenic protein 4 produced in endothelial cells by oscillatory shear stress induces monocyte adhesion by stimulating reactive oxygen species production from a nox1-based NADPH oxidase. *Circ Res* 2004;95:773–779. [PubMed: 15388638]
69. Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2004;24:677–683. [PubMed: 14670934]
70. Azumi H, Inoue N, Ohashi Y, Terashima M, Mori T, Fujita H, Awano K, Kobayashi K, Maeda K, Hata K, Shinke T, Kobayashi S, Hirata K, Kawashima S, Itabe H, Hayashi Y, Imajoh-Ohmi S, Itoh H, Yokoyama M. Superoxide generation in directional coronary atherectomy specimens of patients with angina pectoris: important role of NAD(P)H oxidase. *Arterioscler Thromb Vasc Biol* 2002;22:1838–1844. [PubMed: 12426213]

Abbreviations

LDL	low-density lipoprotein
MM-LDL	minimally modified LDL
ox-LDL	oxidized LDL
ox-PAPC	oxidized 1-palmitoyl-2-arachidonoyl- <i>sn</i> -glycero-3-phosphorylcholine
ROS	reactive oxygen species
G6PD	glucose-6-phosphate dehydrogenase
eNOS	endothelial nitric oxide synthase
BH₄	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

H₂DCFH-DA

2',7'-dichlorodihydrofluorescein diacetate

SiRNA

small interfering RNA

SDS-PAGE

sodium dodecyl sulfate-polyacrylamide gel electrophoresis

DHE

dihydroethidium

RT-PCR

reverse-transcriptase polymerase chain reaction

Fig. 1.

Time-dependent extracellular superoxide ($O_2^{\bullet-}$) production in BAEC measured by cytochrome *c* reduction assay. The rate of $O_2^{\bullet-}$ production increased steadily in response to ox-PAPC compared to control. The addition of SOD decreased the rate of $O_2^{\bullet-}$ production by ~2.5-fold. BAEC incubated with 2-deoxyglucose (2-DOG) at 10 mM showed reduced $O_2^{\bullet-}$ production over 4 h compared to control. The addition of ox-PAPC at 50 μ g/ml to BAEC incubated with 2-DOG induced a rate of $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ was established by parallel measurements in the presence of SOD (60 μ g/ml).

Fig. 2. Intracellular $O_2^{\bullet-}$ production in response to Ox-PAPC with and without PEG-SOD (60 μ g/ml) as visualized by dihydroethidium (DHE) and 2',7'-dichlorofluorescein (2,7-DCF). (a) At time zero, column (a) illustrates BAEC under control, ox-PAPC-, and ox-PAPC + PEG-SOD-treated conditions. (b) Merged images of phase and fluorescence at 4 h demonstrate the localization of red fluorescence in the nuclei. (c) Fluorescent microscopy showed the $O_2^{\bullet-}$ production as red fluorescence. (d) In parallel, ox-PAPC-treated BAEC showed an increase in normalized 2,7-DCF fluorescent intensities over time ($P < 0.05$, $n = 4$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Ox-PAPC-induced up-regulation of Nox4 RNA was inhibited by siNox4. Relative mRNA expression of Nox4 in response to ox-PAPC (50 μ g/ml) and normalized with GAPDH. Ox-PAPC up-regulated relative Nox4 mRNA expression by $3.7\text{-fold} \pm 0.8$ at 4 h ($P < 0.01$, $n = 4$). Pretreatment with siNox4 at 12.5 nM for 6 h resulted in down-regulation of Nox4 mRNA by $80.8 \pm 2.45\%$ at 4 h. Addition of siNox4 to ox-PAPC-treated BAEC also down-regulated Nox4 expression by $60.5 \pm 2.1\%$ at 4 h ($P < 0.01$, $n = 4$). No statistical differences were observed between scrambled siNox4 and siNox4 or between ox-PAPC and scrambled siNox4 + oxPAPC.

Fig. 4.

Extracellular $O_2^{\bullet-}$ production in BAEC measured by cytochrome *c* reduction assay in the presence of inhibitors at 4 h. Addition of NADPH oxidase inhibitor, apocynin, xanthine oxidase inhibitor, allopurinol, and mitochondria complex I inhibitor, rotenone, reduced the rate of $O_2^{\bullet-}$ production by 22.5 ± 3 , 31 ± 4 , and $40 \pm 5\%$, in ox-PAPC-treated BAEC ($P < 0.04$, $n = 4$), respectively, whereas additions of eNOS inhibitor, L-NAME, increased the rates by $15 \pm 3.3\%$ ($P < 0.05$, $n = 4$). The specificity for reduction by $O_2^{\bullet-}$ was established by parallel measurements in the presence of SOD (60 $\mu\text{g/ml}$).

Fig. 5.

Ox-PAPC decreases reduced glutathione in BAEC. GSH levels were determined in BAEC exposed to ox-PAPC (50 μ mg/ml) for 4 h, apocynin (100 μ mol/L), and ox-PAPC plus apocynin. The oxidized form of glutathione (GSSG) was detectable in response to ox-PAPC treatment, but was below the detection limit of the assay in the presence of apocynin with or without ox-PAPC. The ratio of GSH to GSSG is estimated to be 37.0 in the presence of ox-PAPC (GSH = 18.06 ± 0.98 , GSSG = 0.49 ± 0.33 nmol/million cells), suggesting that in the presence of ox-PAPC, GSH was being converted to GSSG in BAEC. There is no statistically significant difference between apocynin and control.

Fig. 6.

The decrease in reducing equivalents produced by ox-PAPC on BAEC is dependent on NADPH as cofactor. The total pool of NADPH and NADH was assayed by fluorescent microscopy revealing a statically significant difference among control, ox-PAPC, 2-DOG, and 2-DOG + ox-PAPC ($P < 0.05$, $n = 4$). Addition of NADPH inhibitor, apocynin, partially restores the NADPH autofluorescence intensity in the ox-PAPC-treated BAEC compared with ox-PAPC-treated sample. Apocynin alone was not statistically significant compared with control.

Fig. 7.

Ox-PAPC regulates MMP-2 and MMP-9 activities. (a) The relative mRNA expression of MMP-2 was up-regulated whereas MMP-9 was down-regulated in BAEC treated with ox-PAPC for 4 h. Both MMP-2 and MMP-9 were normalized with GAPDH. (b) MMP-2 and MMP-9 zymography. The intensities of MMP-2 bands were relatively brighter than those of MMP-9, as expected from their relative mRNA expressions in panel a. (c) SOD attenuated the MMP-2 activity by $25 \pm 10\%$. In the presence of ox-PAPC, MMP-2 activity was increased by $65 \pm 29\%$. The addition of SOD to ox-PAPC-incubated BAEC decreased MMP-2 activity by $30 \pm 23\%$ ($P < 0.05$, $n = 3$). The differences in MMP-9 activities were statistically insignificant (data not shown).

Table 1

Primers and TaqMan probes for bovine Nox4, MMP-2, MMP-9, and GAPDH

Genes	Primer sequences	TaqMan probes
Nox4	5'-AGCAACAAGCCAGTCACCATC-3' 5'-TTCTTTGACCATTCGGATTTCC-3'	5'-(FAM)-GTCACAAGTCATCCGTCAGATGTC-(TAMRA)-3'
MMP-2	5'-CCAAGCTCATCGCCGATG-3' 5'-TGCAGGTCCACCACAGCA-3'	5'-(FAM)-TGGAACGCCATCCCTGATAACCTGG-(TAMRA)-3'
MMP-9	5'-AGCGGAGGACGTCTGCAAC-3' 5'-AGAAATGCAAGCGGTTCTTAATC-3'	5'-(FAM)-ACATCTTCGACGCCATCGCGG-(TAMRA)-3'
GAPDH	5'-CCCCTCCCAACGTGTCTG-3' 5'-ATCTCATCATCTTGGCAGGTTTCT-3'	5'-(VIC)-TGTGGATCTGACCTGCCGCCTG-(TAMRA)-3'