Nrf2 Regulates Antioxidant Gene Expression Evoked by Oxidized Phospholipids in Endothelial Cells and Murine Arteries In Vivo


Abstract—Besides their well-characterized proinflammatory and proatherogenic effects, oxidized phospholipids, such as oxPAPC (oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-phosphocholine) have been shown to have beneficial responses in vascular cells via induction of antioxidant enzymes such as heme oxygenase-1. We therefore hypothesized that oxPAPC could evoke a general cytoprotective response via activation of antioxidative transcription factor Nrf2. Here, we show that oxPAPC increases nuclear accumulation of Nrf2. Using the small interfering RNA approach, we demonstrate that Nrf2 is critical in mediating the induction of glutamate-cysteine ligase modifier subunit (GCLM) and NAD(P)H quinone oxidoreductase-1 (NQO1) by oxPAPC in human endothelial cells, whereas the contribution to the induction of heme oxygenase-1 was less significant. The induction of GCLM and NQO1 was attenuated by reduction of electrophilic groups with sodium borohydride, as well as treatment with thiol antioxidant N-acetylcysteine, suggesting that the thiol reactivity of oxPAPC is largely mediating its effect on Nrf2-responsive genes. Moreover, we show that oxidized phospholipid having a highly electrophilic isoprostane ring in its sn-2 position is a potent inducer of Nrf2 target genes. Finally, we demonstrate that the oxPAPC-inducible expression of heme oxygenase-1, GCLM, and NQO1 is lower in Nrf2-null than wild-type mouse carotid arteries in vivo. We suggest that the activation of Nrf2 by oxidized phospholipids provides a mechanism by which their deleterious effects are limited in the vasculature.

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Key Words: antioxidant response element ■ electrophile response element ■ Nrf2 ■ oxidized phospholipids

Oxidative modification of low-density lipoprotein (LDL) has been implicated to play a role in the atherogenic process. Oxidized phospholipids (oxPLs) present in minimally modified LDL, such as oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-phosphocholine (oxPAPC), contribute to the chronic inflammation characteristic of atherosclerosis. Oxidized phospholipids such as oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-phosphocholine (oxPAPC) have been shown to have antiatherogenic effects of oxPAPC in vascular cells, it has also been shown to have antioxidant enzyme activities. In endothelial cells, oxPAPC induces heme oxygenase (HO)-1, an antiatherogenic protein. OxPAPC also increases the amount of glutathione, an important thiol antioxidant, in endothelial cells. Interestingly, both modifier and catalytic subunits of glutamate-cysteine ligase (GCLM and GCLC, respectively), the rate-limiting enzyme of glutathione synthesis, have recently been shown to be upregulated by oxPAPC in a microarray analysis of human aortic endothelial cells. A common feature of GCLM, GCLC, and HO-1 is that all of these genes have a well-characterized antioxidant response element (ARE), also called electrophile response element.
sequence in their 5’ flanking sequences. The ARE sequence is a regulatory element found in the promoters of a number of antioxidant and phase II detoxification enzymes, and it binds the transcription factor Nrf2, which has recently been reported to mediate the induction of OKL38 gene by oxPAPC. The mechanism of Nrf2-dependent signaling bears similarities with other environmental defense systems, ie, nuclear factor B–mediated inflammatory and hypoxia inducible factor–mediated hypoxic responses. Under basal conditions, Nrf2-dependent transcription is repressed by its negative regulator Keap1, which functions as an adaptor for Cul3-based E3 ligase to facilitate proteasomal degradation of Nrf2. When cells are exposed to oxidative stress or electrophiles, Nrf2 accumulates in the nucleus and drives the expression of its target genes. Although it is evident that Keap1 is a critical negative regulator of Nrf2 signaling and a direct target for its target genes. We aimed to assess the role of Nrf2 in mediating the induction of GCLM, HO-1, and NAD(P)H quinone oxidoreductase-1, another Nrf2-dependent gene important for endothelial antioxidant protection. Moreover, the molecular characteristics of Nrf2-activating oxPLs was examined. Finally, using topical application of oxPAPC to the carotid arteries of either wild-type (WT) or Nrf2–/– animals, we studied the role of Nrf2 in mediating the induction of antioxidant genes in vivo.

Materials and Methods

Details on phospholipids and other reagents, cell culture, cloning of the plasmids, transfection of cells with plasmids or small interfering (si)RNA, real-time quantitative PCR analysis, Western blotting and densitometric quantification, chromatin immunoprecipitation (ChIP), luciferase reporter assay, surgical application of oxPAPC to mouse carotid arteries, immunohistochemistry, and statistical analyses are provided in the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org. All animal procedures were approved by the Experimental Animal Committee of the University of Kuopio.

Results

OxPAPC Activates Nrf2 and Induces HO-1, GCLM, and NQO1

On activation, Nrf2 protein accumulates in the nucleus, which subsequently drives the expression of Nrf2 target genes. We therefore first examined the effect of oxPAPC on nuclear and cytoplasmic content of Nrf2 protein, as well as the expression of HO-1, GCLM, and NQO1, well-characterized Nrf2 target genes. OxPAPC at the concentration of 50 μg/mL increased Nrf2 protein assessed by Western blots in nuclear fractions (Figure 1A). Treatment of human umbilical vein cells (HUVECs) with 0 to 75 μg/mL oxPAPC increased HO-1, GCLM, and NQO1 mRNA and protein concentration dependently (Figure 1B). The fold induction of HO-1 mRNA was notably higher than the other genes, largely because of very low basal expression. The induction of HO-1 by oxPAPC was rapid and preceded the induction of both GCLM and NQO1 (Figure 1C).

Knockdown of Nrf2 Expression by siRNA Inhibits the Induction of HO-1, GCLM, and NQO1 by OxPAPC

To assess as to whether the induction of HO-1, GCLM, and NQO1 is Nrf2-dependent, a specific siRNA against Nrf2 was used. Transfection with Nrf2 siRNA caused a significant inhibition of both Nrf2 mRNA and protein expression in comparison with nonspecific control siRNA (Figure 2A and 2B). Densitometric assessment of nuclear Nrf2 protein content relative to LaminB1 revealed ~90% reduction of Nrf2 protein in cells transfected with Nrf2-siRNA and treated with oxPAPC for 4 hours, in comparison with control siRNA-transfected, oxPAPC-treated cells (Figure 2B). Both basal and the oxPAPC-inducible mRNA expression of GCLM and NQO1 was markedly downregulated by Nrf2 siRNA. However, although the expression of HO-1 mRNA was significantly inhibited by Nrf2 siRNA, the reduction was only ~30%, suggesting that other, redundant pathways in large part mediate the induction by oxPAPC. This notion was further supported by the densitometric analysis of the Western blot data, which shows that oxPAPC-induced expression of GCLM and NQO1 protein expression normalized to β-actin was significantly attenuated by Nrf2-siRNA, whereas HO-1 expression showed a trend toward lower expression, yet did not reach statistical significance (Figure 2C).

OxPAPC Increases the ARE-Driven Gene Expression and Induces Binding of Nrf2 to the ARE Elements of NQO1 and HO-1 Promoter Regions

Nrf2 exerts its effects through binding to the ARE element of the target gene promoter regions. To study whether oxPAPC activates ARE-driven transcription, the luciferase reporter vector containing the ARE element from the human NQO1 promoter was used. A concentration of 50 μg/mL oxPAPC increased ARE-driven transcription assessed by increased luciferase activity, whereas in the construct in which the consensus ARE element was mutated, both basal and inducible activity was attenuated (Figure 3A). On exposure to oxPAPC, there was minor residual activation of the empty pGL3-basic vector and the vector having the NQO1 core ARE mutated. The vector backbone of pGL3-luciferase expression vector has several putative consensus binding sites for transcription factors, which may be responsive to oxPAPC. For example, it contains a consensus activator protein-1 site, which may impact the activity of the construct, because activator protein-1 has been shown to be activated by oxPAPC. However, the residual activity was very low and the direct binding of Nrf2 to the NQO1 promoter on exposure to oxPAPC was verified by ChIP assay (Figure 3B). Treatment of HUVECs with oxPAPC also increased the binding of Nrf2 to the distal enhancer region of HO-1 gene containing multiple AREs (Figure 3B). These results demonstrate that oxPAPC activates ARE and increases the binding of Nrf2 to both NQO1 and HO-1 ARE elements.
Figure 1. OxPAPC induces nuclear accumulation of Nrf2 and the expression of HO-1, GCLM, and NQO1 in a concentration and time-dependent manner. A, Nuclear and cytoplasmic Nrf2 protein detected by Western blotting in HUVECs after treatment with 50 μg/mL oxPAPC. Lamin B1 was used for control of nuclear proteins. B, HUVECs were treated with 0 to 75 μg/mL oxPAPC for 8 hours for RNA analyses by quantitative real-time PCR (bar graphs) or 16 hours for protein (Western blots). 1,2-Dimyristoyl-sn-3-glycerophosphocholine (DMPC) (100 μg/mL) was used as a nonoxidized phospholipid control. C, HUVECs were treated with 50 μg/mL oxPAPC for 0 to 24 hours, and the mRNA and protein expressions were studied as above. The mRNA expression in B and C is normalized to ribosomal RNA (rRNA) and depicted as fold change vs untreated control±SEM (n=3). *P<0.05 in comparison with untreated controls. The Western blots are representative of 3 independent experiments.
Characterization of the Lipid Species Inducing Antioxidant Enzymes

As shown in Figure 2, the induction of GCLM and NQO1 by oxPAPC was critically dependent on Nrf2, indicating that the expression of these genes could be used as a readout for the Nrf2-activating effects of different classes of oxPLs. To study the effect of polar head groups in sn-3 position on Nrf2 activation, cells were stimulated by oxPLs containing identical sn-1 and sn-2 residues (palmitoyl and arachidonoyl, respectively) but different polar head groups (for a structure of PAPC, see Figure 1 in the online data supplement). Replacement of oxPAPC with oxidized phosphatidylglycerol, phosphatidic acid, phosphatidylethanolamine, or phosphatidylserine yielded similar upregulation of GCLM and NQO1 mRNA (Figure 4A), suggesting that the polar head group in sn-3 residue does not play an important role in activity. Next, the significance of the sn-2 residue was examined. We found that the presence of oxidized sn-2 residue was an absolute prerequisite for activation of GCLM and NQO1 genes because neither unoxidized phospholipids nor 1-palmitoyl-2-
hydroxy-sn-3-glycerophosphocholine (lysoPC), in which only a hydroxy group in sn-2 position is present, were active (Figure 4A and 4B).

Oxidation of PAPC yields phospholipids containing oxidatively fragmented sn-2 residues such as 1-palmitoyl-2-(5-oxovaleroyl)-sn-3-glycerophosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-sn-3-glycerophosphocholine (PGPC) (supplemental Figure I). We next examined the effect of these on the mRNA expression of GCLM and NQO1. Exposure of HUVECs to POVPC or PGPC did not increase the expression of either gene, indicating that these were reduced by sodium borohydride (NaBH4). Treatment with NaBH4 almost completely abolished the effect of isoprostane-PC on the expression of GCLM and NQO1 genes. Incubation with NAC inhibited the induction of Nrf2 target genes by PAPCOOH and oxPAPC with NaBH4 partially suppressed its ability to induce GCLM and NQO1 in HUVECs (Figure 5A). Moreover, coincubation with nucleophilic thiol antioxidants N-acetylcysteine (NAC) and glutathione reduced the induction of HO-1, GCLM, and NQO1 by both species (Figure 5B and supplemental Figure IV). We also examined the effect of NAC on the induction of Nrf2 target genes by PAPCOOH and isoprostane-PC. Incubation with NAC inhibited the induction of HO-1, GCLM, and NQO1 by both species (Figure 5C and 5D). Moreover, treatment with NaBH4 almost completely abolished the effect of isoprostane-PC on the expression of these genes (Figure 5D). To conclude, these data indicate that the electrophilic character of oxPAPC and its active components are largely mediating the effect on Nrf2-responsive genes, but additional mechanisms may be required for maximal induction of these genes by oxPLs.

**Figure 3.** OxPAPC activates ARE and induces binding of Nrf2 to the ARE elements of NQO1 and HO-1. A, HUVECs were transfected with empty pGL3-promoter vector, vector containing the NQO1-ARE, or mutated NQO1-ARE. Transfected cells were treated with 50 µg/mL oxPAPC for 16 hours. Results were normalized to total protein and presented as relative activity vs empty vector. B, HUVECs were treated with 50 µg/mL oxPAPC for 3 hours. The binding of Nrf2 to the promoter regions of HO-1 and NQO1 was determined with ChIP. HO-1 exon3 was used as a negative control. Samples are representative of 3 independent experiments.

**Figure 4.** A, Comparison of the in vivo induction of HO-1, GCLM, and NQO1 in mouse carotid arteries by oxPAPC and oxPLPC. The expression of HO-1, GCLM, and NQO1 in vivo was measured by reverse transcription PCR. B, HUVECs were treated with 50 µg/mL oxPAPC for 3 hours. The binding of Nrf2 to the promoter regions of HO-1 and NQO1 was determined with ChIP. HO-1 exon3 was used as a negative control. Samples are representative of 3 independent experiments.

**Figure 5.** OxPAPC induces HO-1, GCLM, and NQO1 through an Nrf2-dependent mechanism in murine arteries in vivo. Finally, we wanted to examine the role of Nrf2 in mediating the expression of HO-1, GCLM, and NQO1 in vivo. To this end, oxPAPC in pluronic gel was applied to the adventitial side of surgically exposed carotid arteries of either WT C57BL/6 controls or Nrf2-KO mice as in. The concentration used corresponds to concentrations of isoprostane-PC and other bioactive phospholipids that are lower than those found in the aortas of rabbits fed the atherogenic diet. Mouse carotid arteries consist of only 4 to 5 cell layers, allowing penetration of oxPAPC through the vessel wall. We first studied the mRNA expression of HO-1, GCLM, and NQO1 in mouse carotid arteries using quantitative real-time
PCR. After 6 hours of treatment with oxPAPC, all 3 genes were significantly upregulated in WT mouse carotid arteries in comparison with controls treated with pluronic gel only (Figure 6A). The induction was completely abolished in the arteries of Nrf2-KO animals (Figure 6A). In addition, the basal expression of NQO1 was also significantly lower in Nrf2-KO mice compared with WT controls.

We next examined the HO-1 and NQO1 protein expression in mouse carotid arteries in WT and Nrf2-KO mice using immunohistochemistry. Immunohistochemical analysis of mouse carotid arteries exposed to oxPAPC for 24 hours showed increased HO-1 expression in oxPAPC-treated arteries in WT but not in Nrf2-KO arteries (Figure 6B). HO-1-positive cells were localized mainly in the adventitia of the vessels (Figure 6B). The expression NQO1 protein was more uniformly increased throughout the vessel wall in oxPAPC-treated WT carotid arteries, with increased positive staining in the adventitial and medial layers and especially in the endothelial layer (Figure 6C). In the Nrf2-KO mouse arteries, exposure to oxPAPC did not increase NQO1 positive staining (Figure 6C).

**Discussion**

There are a number of reports showing in vitro that Nrf2 could have antiinflammatory effects in vascular cells. In cultured endothelial cells, Nrf2 is activated by shear stress, a potent antiinflammatory force.18,19 Overexpression of Nrf2 downregulates the tumor necrosis factor-α-induced transcriptional increase in vascular cell adhesion molecule-1 expression and inhibits monocyte adhesion to the endothelium.18,26 Furthermore, Nrf2 has been shown to mediate adaptive augmentation of antioxidant defenses of vascular cells on exposure to a variety of lipid oxidation products such as oxidized LDL, a lipid-derived aldehyde 4-hydroxynonenal, or cyclopentenone prostaglandins and isoprostanes.9,15,27,28 Herein, we expand these previous studies to show that also oxPAPC can evoke a concerted Nrf2-mediated response. Moreover, we show in vivo using WT and Nrf2-null mice that oxPAPC upregulates the expression of HO-1, GCLM, and NQO1 mRNA and HO-1 and NQO1 protein in mouse carotid arteries in WT but not in Nrf2-KO mice, demonstrating the role of Nrf2 in regulating these genes also in intact vessels. Inasmuch as we have recently shown in rabbit balloon injury model that adenoviral Nrf2 gene transfer can attenuate injury-induced vascular inflammation, as well as inhibit the accumulation of oxidized LDL in the vessel wall,29 it is conceivable that the Nrf2 response provides a mechanism by which the deleterious effects of oxPAPC are limited.

In the present study, the induction of HO-1 in vivo in mouse carotid vessels appeared to be critically dependent on Nrf2, whereas in human endothelial cells, suppression of Nrf2 expression by siRNA had a markedly smaller impact on HO-1 expression than on the other target genes GCLM and NQO1. Although both genes have been shown to contain the 2 enhancer regions in their promoters with multiple ARE binding sites, this does not unequivocally mean that the regulation of mouse and human genes is identical. Although our ChIP results clearly indicate that Nrf2 binding is increased in distal enhancer region in the HO-1 gene on oxPAPC exposure, it is evident that other, redundant pathways are involved in HO-1 induction. For example, in previous studies, cAMP-responsive element-binding protein,6
was shown to be involved in the induction of HO-1 by oxPAPC in human endothelial cells. Also a number of other transcriptional regulators and signaling pathways, such as nuclear factor-κB and PPRE (peroxisome proliferator-activated receptor response element), contribute to the regulation of HO-1,7,8 highlighting the complexity of HO-1 regulation in different cell types and by different stimuli. It is also noteworthy that in our study, the induction of HO-1 in vivo is markedly lower than the up to 100-fold induction achieved in vitro, making it likely that in the latter case redundant pathways contributing to the activation are needed for a sufficient response.

At present, the cellular receptor mediating the effect of oxPAPC on Nrf2 activation remains unclear. It also remains an open question whether the Nrf2 activating capacity of oxPLs can be modified by phospholipases, eg, by phospholipase A2 catalyzing the cleavage at the sn-2 position, shown to reduce the proinflammatory activity of oxPAPC.25 It is possible that intact electrophilic oxPLs could directly bind to intracellular Keap1, because phospholipids can be taken up by the cell by transbilayer movement,30,31 or by receptor-mediated mechanisms.32 Keap1 has highly reactive cysteine residues, which can be modified by direct alkylation by electrophiles, including 15-deoxyΔ12,14-prostaglandin J2.15,19 Interestingly, oxPAPC is known to contain esterified cyclopentenone isoprostanes.22 Also, it has recently been demonstrated that oxidized phospholipids can have intracellular targets33 and that they can covalently bind to intracellular signaling proteins, such as H-Ras.34 However, oxPAPC may also have targets at the cell surface which could evoke the Nrf2 response. Several cell surface receptors have been proposed to be receptors for oxPLs, including PAF-receptor,35 lysophospholipid receptors such as G2A,36 Toll-like receptor 4,37 and prostaglandin E2 receptor subtype 2.38 However, the structure–function relationship found in our experiments does not support the involvement of any of these in Nrf2 activation.

It has been proposed that exposure to oxPAPC leads to activation of NAD(P)H oxidase and production of reactive oxygen species in endothelial cells, mediating downstream effects on gene expression.12,39 Regarding the role of NAD(P)H oxidase in Nrf2 activation, there are a few reports showing a connection.40–42 Unfortunately, these reports use diphenyleneiodonium to inhibit NAD(P)H oxidase. This compound is not a specific NAD(P)H oxidase inhibitor but inhibits flavoenzymes in general, some of which (eg, NQO1, glutathione reductase) are antioxidant enzymes and Nrf2 target genes. Therefore the effects of diphenyleneiodonium are difficult to interpret. We have done experiments in which we look at the effect of NAD(P)H inhibitor apocynin, as well as siRNA specific to Nox4, the major component of endothelial NADPH oxidase,43 which has also been reported to be responsive to oxPAPC.39 Neither apocynin nor Nox4 siRNA had any impact on Nrf2 target gene expression (results not shown). These results support the notion that secondary ROS...
production by NAD(P)H oxidase is not necessary for Nrf2 activation.

In summary, our study has identified molecular mechanisms by which oxPLs induce antioxidant genes and shows the critical dependence on Nrf2 in the induction of HO-1, GCLM, and NQO1 expression by oxPAPC in vivo. We postulate that the activation of Nrf2 by oxPLs provides a mechanism by which their proatherogenic effects are limited in the vessel wall.

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Disclosures
None.

References


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**Materials and Methods**

**Lipids**

Synthetic 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoglycerol (PAPG), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphate (PAPA), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoserine (PAPS), 1-palmitoyl-2-hydroxy-sn-3-glycero-phosphocholine (lysoPC), and 1-palmitoyl-2-linoleoyl-sn-3-glycerophosphocholine (PLPC) were purchased from Avanti Polar Lipids. Arachidonic acid was from Sigma. Dry lipids were oxidized by exposure to air until approximately 80% of the lipid was oxidized. Oxidized lipids were dissolved in chloroform, purged with argon and stored at -70°C. Oxidation was monitored by thin-layer chromatography and electrospray ionization-mass spectrometry.\(^1\) 1-Palmitoyl-2-(5,5’-dimethoxyvaleroyl)-sn-3-glycero-phosphocholine (acetal-POVPC), 1-palmitoyl-2-(5-oxovaleroyl)-sn-3-glycero-phosphocholine (POVPC), and 1-palmitoyl-2-glutaroyl-sn-3-glycero-phosphocholine (PGPC) were synthesized from lysoPC according to the previously published protocol,\(^1\) except for using dicyclohexylcarbodiimide immobilized on polymer beads (Novabiochem). 1-Palmitoyl-2-(5,6-epoxy isoprostane E2)-sn-3-glycero-phosphocholine (isoprostane-PC)-enriched fraction was isolated from oxPAPC by HPLC purification on a Adsorbosphere Si 100 column (5 µm, 250 × 10 mm, Alltech) using isocratic elution with a mixture of acetonitril/methanol/water (77:8:15, v/v) supplemented with 0.02 vol % of triethylamine at the flow rate of 3 mL/min. A mixture of 1-palmitoyl-2-(12-hydroperoxyeicosa-5,8,10,14-tetraenoyl)-sn-3-glycerophosphocholine and 1-palmitoyl-2-(15-hydroperoxyeicosa-5,8,11,13-tertraenoyl)-sn-3-glycerophosphocholine (PAPC-OOH) was produced from PAPC by enzymatic reaction with a soybean lipoxygenase.\(^2\) PAPC-OOH was purified on solid phase extraction cartridge using a previously published protocol.\(^3\) Measurement of lipid peroxides was performed using a ferric thiocyanate method.\(^4\) Hydroxide of PAPC (PAPC-OH) was obtained from PAPC-OOH by
reduction with triphenylphosphine. Reduction of oxPAPC with sodium borohydride was performed as follows. A solution of sodium borohydride in isopropanol (2.3 mL, 1.73 mmol/L) was added to oxPAPC (1 mg) dried under the argon stream in a glass tube. The mixture was vortexed vigorously and incubated for 1 hr at 0°C. The excess of NaBH₄ was destroyed by careful addition of aqueous HCl (0.1 mol/L). The lipids were extracted with chloroform (2 × 10 mL). The lower organic layer was evaporated under reduced pressure. The residue was applied on an HPLC column and the phospholipids were eluted as described above. Concentration of phospholipids was determined by phosphorus assay. Before adding to cells, the lipids were resuspended by vigorous vortexing in the medium supplemented with 2% FCS.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) isolated from umbilical chords obtained from the maternity ward of the Kuopio University Hospital, by the approval of the Kuopio University Hospital Ethics Committee, were cultivated as described previously.

Cloning of Plasmids

For cloning pGL3-NQO1-ARE and pGL3-NQO1-AREmut constructs specific oligonucleotides containing KpnI and SacI (NQO1-ARE) or NheI and XhoI (NQO1-AREmut) restriction sites were synthesized (TAG Copenhagen). The sequences of the sense strands of NQO1-ARE and NQO1-AREmut were 5' CGATCCAGTCACAGTGACTCAGCAGAATCTGGAGCT 3' and 5' CTAGCGATCCAGTCACAGTAACCTCAGCAGAATCTGC 3' respectively. Complementary oligonucleotides were annealed and ligated into respective restriction sites of the pGL3-promoter vector (Promega) and verified by sequencing.

SiRNA Transfection
Small interfering RNA (siRNA) oligonucleotide targeting Nrf2, and a non-specific RNA control were obtained from Dharmacon (ON-TARGETplus SMARTpool siRNA reagent). HUVECs were seeded on 6-well plates at the density of 150 000 cell/well and allowed to adhere for 24 h. Cells were transfected with 100 nmol/L siRNA oligonucleotides using 2 μl of Oligofectamine (Invitrogen) in 1 ml of media. Assessed by rhodamine-labeled siRNA and fluorescence microscopy, this protocol yielded ca. 90% transfection efficiency in HUVECs, with no toxic effects assessed by morphology and LDL release. 24 h after transfection, cells were treated with phospholipids for 6 to 16 h for quantitative real time PCR and Western blot analyses.

**RNA Isolation and Quantitative Real-time PCR**

RNA was extracted with Trizol-reagent (Invitrogen) and 1 μg of total-RNA was used for the cDNA synthesis using random hexamer primers (Promega) and M-MuLV reverse transcriptase (New England Biolabs). The relative expression levels of mRNA encoding Nrf2, HO-1, GCLM or NQO1 in HUVECs as well as HO-1, GCLM and NQO1 in mouse carotid arteries were measured according to manufacturer’s protocol by quantitative real time PCR (ABI PRISM 7700 Sequence detector, Applied Biosystems) using specific Assays-on-demand (Applied Biosystems) target mixes. The expression levels were normalized to the level of ribosomal RNA or β2-microglobulin (Applied Biosystems).

**Western Blotting and Densitometric Quantification**

Cells were lysed and total protein concentration was measured with BCA-assay (Pierce). For isolation of nuclear and cytoplasmic fractions, these were isolated with NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce). 10 to 20 μg of protein was used for electrophoresis. The proteins were transferred to nitrocellulose membrane, blocked and incubated with primary antibodies. The primary antibodies used were rabbit polyclonal anti-Nrf2 (Santa Cruz), rabbit
polyclonal anti-HO-1 (Stressgen), anti-GCLM (a gift from Dr. Terrance Kavanagh, University of Washington, Seattle, WA), mouse monoclonal anti-NQO1 (Clones A180 and B771, gifts from Dr. David Ross, University of Colorado, Denver, CO), rabbit polyclonal anti-β-actin (Cell Signaling), and rabbit polyclonal anti-Lamin B1 (Abcam). Blots were visualized using HRP-conjugated secondary antibodies and Supersignal™ chemiluminesence substrate (Pierce). Blots were visualized using ECL Plus Western Blotting Detection System with Typhoon 9400 (GE Healthcare). Protein expression data was quantified with ImageQuant TL 7.0 software (GE Healthcare).

**Luciferase reporter assay**

Activation of ARE by oxPAPC or subfractions was measured using Luciferase Reporter Assay (Promega) from HUVECs transfected with an empty pGL3-promoter vector, NQO1-ARE or NQO1-AREmut luciferase reporter plasmids as in.8 24 h after transfection, the cells were treated with 50 µg/mL oxPAPC. After 16 h, the cells were collected and analyzed as described.8

**Chromatin immunoprecipitation (ChIP)**

HUVECs grown on 10-cm dishes were treated with 50 µg/mL oxPAPC for 3 hours. DNA and proteins of treated cells were cross-linked by incubating cells in 1 % formaldehyde for 10 min at room temperature. Cross-linking was stopped by 10 min incubation with 0.125 mol/L glycine. Cells were washed with cold PBS and lysed with 1 ml ChIP- lysis buffer (50 mmol/L HEPES-KOH, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 140 mmol/L NaCl, 10% glycerol, 0.5 % Nonidet-P40, 0.25% Triton X-100) with protease inhibitors (Roche). Lysates were incubated 10 min on ice and centrifuged (700 g, 5 min, +4 °C) to pellet the nuclei. Pellets were resuspended in 1 mL ChIP wash buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 200 mmol/L NaCl) with protease inhibitors and incubated for 10 min on ice. After centrifugation, pellets were suspended in 1 mL ChIP-RIPA-buffer (10 mmol/L Tris-HCl; 1 % Triton X-100; 0.1 % SDS; 0.1 %
sodium deoxycholate; 1 mmol/L EDTA; 0.5 mmol/L EGTA; 140 mmol/L NaCl) with protease inhibitors. Chromatin was sonicated to 200 to 1000 bp fragments on ice. Nuclear debris was removed by centrifugation (16 100 g, 15 min, 4 °C) and samples were pre-immunoprecipitated with rabbit serum (Vector laboratories) and Protein A-Sepharose beads (Amersham Bioscience) for 2 h at 4 °C. After centrifugation, 100 µL of each sample was separated for input control and the remaining sample was immunoprecipitated with 3 µg of specific Nrf2 antibody (Santa Cruz Biotechnology), non-specific immunoglobulin G (anti-rabbit IgG, Vector laboratories) or H2O overnight at 4 °C. Immune complexes were precipitated with Protein A-Sepharose beads for 2 h at 4 °C. Sepharose beads were washed twice with RIPA buffer, once with TSE I-, TSEII- and LiClBuffers and three times with TE-buffer. DNA-protein complexes were eluted from Protein A-Sepharose beads with elution buffer (1% SDS, 100 mmol/L NaHCO3). Cross-linking was reversed at 65 °C overnight and DNA was extracted using QiAamp DNA mini kit (QIAGEN) according manufacturer's protocol for cultured cells. PCR were performed against the ARE element of HO-1 (primers 5'-CCATCTGGCGCCGCTCTGC-3' and 5'-GAGCAGCTGGAACTCTGAGGA-3') or NQO1 (primers 5'-AAGTGTGTGTTGTATGGGGCCC-3' and 5'-TCGTCCCAAGAGAGTCAGG-3') promoters as well as a nonspecific binding site from HO-1 exon3 (primers 5'-ATCTATGTGGCCCTGGAGGAG-3' and 5'-TCGTGGAGCCGCTTCACATAG-3'). PCRs were performed in 25-µL reaction mixtures containing 25 pmol of each primer, 200 µmol/L deoxynucleoside triphosphate (dNTP, Finnzymes Diagnostic), 0.8 U Dynazyme DNA polymerase (Finnzymes Diagnostic), 2 mmol/L MgCl2 (Finnzymes Diagnostic) and 2.5 µL 10x reaction buffer (Finnzymes Diagnostic). Initial denaturation (5 min at 95°C) was followed by 40 cycles (HO-1, nonspecific control) or 50 cycles (NQO1) for 30s at 95°C, for 30s at 60°C (HO-1, nonspecific control) or 59°C (NQO1), and for 30s at 72°C. PCR was completed by 10 min at 72°C and 1 min at 95°C and products were separated on 1.2 % agarose gel.
Application of OxPAPC to Carotid Arteries

The application of oxPAPC dissolved in pluronic gel to mouse carotid arteries was done as previously described. Briefly, animals were anesthetized using ketamine (Ketalar, Pfizer) and xylazin (Rompun Vet, Bayer) and the local anesthesia was obtained using Lidocaine (Lidocain, Orion Pharma). Left carotid artery was exposed through midline cervical incision and 60 μl of 30 % Pluronic gel with or without 50 μg oxPAPC was applied on carotid artery. The wound was closed with 5-0 sutures. Wild type or Nrf2-knockout mice backcrossed to C57Bl/6 background (6 mo of age) were used for the study. The animals were sacrificed with carbon dioxide and carotid arteries were collected 6 h after the surgery for mRNA analysis with quantitative real time PCR, or 24 h after the surgery for immunohistochemistry.

Immunohistochemistry

The carotid arteries were immersion-fixed in 4% phosphate-buffered paraformaldehyde for 4 hours, rinsed in 15 % saccharose overnight and embedded in paraffin and cut in 6 μm sections. Sections were stained against rabbit polyclonal antibodies against HO-1 (Stressgen) and NQO1 (Abcam). An avidin-biotin-horseradish peroxidase system (Vector Laboratories) and DAB-plus kit (Zymed Laboratories) were used for signal detection. Control immunostainings were conducted without primary antibody. Olympus AX-70 (Olympus Optical) microscope and AnalySIS-software (Soft Imaging Systems) were used for analyses of the samples.

Statistical analyses

Statistical analyses were performed with GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA). Results are expressed as mean±SEM. Statistical significance was evaluated by paired t-test, and p<0.05 was considered statistically significant.
References


**Supplemental Figure I:** Chemical structures of PAPC and select biologically active oxidized phospholipids.
**Supplemental Figure II:** Reduction of carbonyl and hydroperoxide groups by TPP and NaBH$_4$. 
Supplemental Figure III. Representative mass-spectra of mock-treated (A) and NaBH₄-reduced (B) oxPAPC. Chemical reduction was performed as described in the supplementary methods section. The lipids were analyzed by electrospray ionization/mass-spectrometry in the positive mode.
Supplemental Figure IV. Inhibition of HO-1, GCLM, and NQO1 expression by GSH. Cells were exposed to 50 µg/mL oxPAPC for 16 h in the presence and absence of NAC or GSH and the protein expression of HO-1, GCLM, and NQO1 was measured by Western blot, using β-actin as a loading control. The blots are representative of three independent experiments.

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