Cell Biology/Signaling

Oxidized Phospholipids Regulate Expression of ATF4 and VEGF in Endothelial Cells via NRF2-Dependent Mechanism: Novel Point of Convergence Between Electrophilic and Unfolded Protein Stress Pathways

Taras Afonyushkin, Olga V. Oskolkova, Maria Philippova, Therese J. Resink, Paul Erne, Bernd R. Binder, Valery N. Bochkov

Objective—The ATF4 arm of the unfolded protein response is increasingly recognized for its relevance to pathology, and in particular to angiogenic reactions. Oxidized phospholipids (OxPLs), known to accumulate in atherosclerotic vessels, were shown to upregulate vascular endothelial growth factor (VEGF) and induce angiogenesis via an ATF4-dependent mechanism. In this study, we analyzed the mechanism of ATF4 upregulation by OxPLs and more specifically the involvement of NRF2, the major transcriptional mediator of electrophilic stress response.

Methods and Results—Using reverse transcription/real-time polymerase chain reaction and Western blotting, we found that OxPLs induced upregulation of ATF4 mRNA and protein in several types of endothelial cells and that these effects were suppressed by short interfering RNA (siRNA) against NRF2. Electrophilic (iso)prostaglandins and oxidized low-density lipoprotein, similarly to OxPLs, elevated ATF4 mRNA levels in an NRF2-dependent mode. Chromatin immunoprecipitation revealed OxPL-dependent binding of NRF2 to a putative antioxidant response element site in the ATF4 gene promoter. Knockdown of NRF2 inhibited OxPL-induced elevation of VEGF mRNA and endothelial cell sprout formation.

Conclusion—Our data characterize NRF2 as a positive regulator of ATF4 and identify a novel cross-talk between electrophilic and unfolded protein responses, which may play a role in stress-induced angiogenesis. (Arterioscler Thromb Vasc Biol. 2010;30:1007-1013.)

Key Words: oxidized phospholipids ■ NRF2 ■ ATF4 ■ vascular endothelial growth factor ■ angiogenesis

Oxidation of unsaturated fatty acids esterified in phospholipids generates a group of mediators demonstrating multiple biological effects, including proinflammatory, thrombogenic, antiendothelin, and immunomodulating activities. Previously, we characterized the angiogenic activity of oxidized phospholipids (OxPLs) and showed that induction of vascular endothelial growth factor (VEGF) is an important, although not exclusive, mechanism of angiogenic switch initiated by OxPLs. The analysis of signaling pathways activated by OxPLs identified ATF4 as the major regulator of VEGF expression in OxPL-treated cells. ATF4 is a key transcriptional effector of cellular stress response generally known as unfolded protein response (UPR), which is induced by accumulation of improperly folded proteins. In addition, ATF4 is upregulated by deficit of amino acids, oxidative stress, and other deleterious conditions, and therefore the ATF4 branch of UPR is often referred to as integrated stress response. The ATF4 pathway is initiated by stress-activated protein kinases, eg, eukaryotic translation initiation factor 2alpha kinase 3 (PERK), which phosphorylates eukaryotic translation initiation factor 2, subunit 1 (eIF2α), leading to general inhibition of protein synthesis but selective translation of ATF4.

A separate stress pathway that protects cells from chemically reactive compounds is the electrophilic stress response (ESR). Canonical activation of ESR is initiated through oxidative modification of KEAP-1 by electrophiles, leading to dissociation of NRF2/KEAP-1 complexes and enhanced transcription of genes having NRF2-binding antioxidant response elements (AREs) in their promoter regions, eg, heme oxygenase-1, known for its antioxidant, antiinflammatory, and proangiogenic properties. In addition to sensing electrophilic stress, NRF2 is thought to be of key importance for antioxidant and antiinflammatory effects of shear stress in atherosclerosis-resistant areas of vessels. However, the general role of NRF2 in atherogenesis may be more complex, because knockout of Nrf2 inhibits development of atherosclerosis.

OxPLs were shown previously to activate both ATF4- and NRF2-dependent transcription. Activation of NRF2 may...
be explained either by direct electrophilic attack of reactive OxPL species or by activation of enzymes producing oxidizing and electrophilic molecules, eg, NADPH oxidase or endothelial NO synthase.\textsuperscript{17,18} the detailed mechanisms of ATF4 upregulation by OxPLs are not known. In this work, we analyzed intracellular signaling mechanisms whereby OxPLs stimulate expression of ATF4, leading to an angiogenic switch. Unexpectedly, we found that induction of ATF4 by OxPLs significantly depends on NRF2 activity. These data point to the existence of a previously unidentified link between UPR and ESR pathways and suggest that NRF2 can promote pathological effects induced via ATF4.

**Experimental Procedures**
A detailed description of materials and methods is presented in the Supplemental Data, available online at http://atvb.ahajournals.org.

**Lipids**
Arachidonate-containing synthetic lipids were purchased from Avanti Polar Lipids. Dry lipids were oxidized by exposure to air and analyzed by mass spectrometry.\textsuperscript{19} The dried lipids were resuspended by vigorous vortexing in medium 199 supplemented with 2% FCS before use in cell culture experiments.

**mRNA and Protein Analysis**
mRNAs levels were quantified by reverse-transcription/quantitative real-time polymerase chain reaction and were normalized to \(B_{2}\)-microglobulin mRNA levels.

**Western Blotting**
Protein samples were separated on SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membrane by electroblotting. Membranes were probed with rabbit antibodies against ATF4, NRF2, PERK, phospho-eIF2\(\alpha\), total eIF2\(\alpha\), \(\beta\)-actin, and peroxidase-conjugated secondary antibody.

**RNA Interference**
Cells were transfected with 100 nmol/L of short interfering RNA (siRNA) using polyethylenimine (PEI) reagent.\textsuperscript{20} Efficiency of silencing was checked at the mRNA and/or protein level.

**Chromatin Immunoprecipitation**
Chromatin immunoprecipitation analysis was performed using EZ-Chip kit (Upstate Biotechnology). Purified immunoprecipitated and input DNA was analyzed by polymerase chain reaction. Polymerase chain reaction products were resolved on polyacrylamide gels and stained with ethidium bromide.

**NRF2 Binding Competition Assay**
NRF2-DNA interactions were also characterized using the ELISA-based TransAM NRF2 kit (Active Motif). Nuclear extracts from untreated human umbilical vein endothelial cells (HUVECs) and cells stimulated with oxidized 1-palmityl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) were prepared using the NE-Per kit (Pierce).

**Spheroid Assay of Angiogenesis In Vitro**
After transfection with siRNA, cell spheroids were formed and embedded in fibrin gels as described.\textsuperscript{2} Polymerized gels were overlaid with medium 199 supplemented with 2% FBS with or without inclusion of OxPAPC (130 \(\mu\)mol/L). After 24 hours, gels were fixed and actin structures were visualized with tetramethylrhodamine B isothiocyanate-conjugated phalloidin (Sigma-Aldrich).

**Statistical Analysis**
The data are representative for 2 to 4 independent experiments and are expressed as mean±SD of triplicate or quadruplicate measurements. The analysis was performed by the 2-tailed Student \(t\) test. A probability value below 0.05 was considered as significant.

**Results**

**OxPLs Upregulate ATF4 mRNA Levels**
The classic mechanism of ATF4 upregulation during UPR is based on selective translation resulting from phosphorylation of eIF2\(\alpha\) by PERK.\textsuperscript{21} Our previous kinetic measurements showed that the levels of eIF2\(\alpha\) phosphorylation and ATF4 protein in HUVECs started to increase after 1 to 2 hours of treatment with OxPAPC.\textsuperscript{2} In this study, we characterized an additional mechanism of ATF4 regulation by OxPLs. OxPAPC induced an elevation of ATF4 mRNA that began after 1 hour and was sustained for at least 6 hours after addition of the lipid to HUVECs (Figure 1A). Thus, elevation of ATF4 mRNA coincided with the time course of phosphorylation of eIF2\(\alpha\), expected to induce selective translation of ATF4. Therefore, elevated levels of ATF4 mRNA are likely to contribute to the increased synthesis of ATF4 protein.

Elevation of ATF4 mRNA induced by OxPAPC in HUVECs (Figure 1B) developed within the range of concentrations previously shown to induce ATF4-dependent elevation of VEGF and stimulation of angiogenic reactions.\textsuperscript{2,3} Human coronary artery and uterine microvascular endothelial cells were also sensitive to OxPAPC, demonstrating statistically significant elevation of ATF4 mRNA at concentrations above 3 \(\mu\)mol/L (Figure 1C). OxPLs with different head groups induced comparable elevation of ATF4 mRNA (Figure 1B), which agrees with previous findings that different classes of OxPLs can elevate ATF4 protein and its downstream target VEGF.\textsuperscript{2,3} These data further support our hypothesis that oxidized fatty acid residues rather than polar head groups are critical for the angiogenic activity of OxPLs,\textsuperscript{2,3} in turn suggesting that different OxPL classes formed in vivo can induce an angiogenic switch in an additive manner.

Elevation of ATF4 mRNA levels was induced by representative and abundant molecular species of OxPCs containing fragmented and full-length oxidized sn-2 residues (Figure ID), further suggesting that the combined concentration of all active classes and molecular species of OxPLs may be of higher biological relevance than levels of individual species. Because the most active of these species contained electrophilic groups, we tested whether the induction of ATF4 mRNA may be modulated by OxPLs via the NRF2 pathway characteristic of ESR.\textsuperscript{15}

**NRF2 Is Involved in OxPAPC-Induced Upregulation of ATF4 mRNA and Angiogenic Reactions**
In support of our hypothesis about involvement of the NRF2 pathway in upregulation of ATF4 mRNA, we found that reduction of electrophilic groups in OxPAPC by sodium borohydride resulted in statistically significant attenuation of ATF4 mRNA elevation (Figure 2A). Thus,
electrophilic properties of OxPLs seem to be important
determinants of their ability to increase ATF4 mRNA.
Typical inducers of electrophilic stress, such as butylated
hydroxyanisole and cyclopentenone (iso)prostaglandins,
also elevated levels of ATF4 mRNA (Figure 2A), further
supporting the role of NRF2 as a positive modulator of the
ATF4 pathway.

Pretreatment of cells with siRNA against NRF2, but not
with scrambled siRNA, consistently resulted in inhibition
of OxPAPC-induced upregulation of ATF4 mRNA, as well
as glutamate-cysteine ligase, modifier subunit (GCLM),
which is a classic NRF2-activated gene (Figure 2B; Sup-
plemmental Figures I and III), siRNA targeting a nonover-
lapping site in NRF2 mRNA produced a comparable
decrease in OxPAPC-induced ATF4 mRNA elevation in
HUVECs, from 6.53±3.40-fold to 1.94±1.17-fold
(P<0.036), pointing to the specificity of the effect of
NRF2 silencing. The effect did not result from toxic action
of lipids or siRNA, because OxPAPC either alone or after
the transfection procedure did not significantly influence
the levels of housekeeping protein actin in 3 endothelial
cell types 48 hours after transfection (Figure 3A and 3B),
nor induction by OxPAPC of other genes, eg, COX-2
(Supplemental Figure IV). Furthermore, mRNAs encod-
ing ATF4 and classic electrophilic genes GCLM and
GCLC were upregulated by oxidized (but not native)
low-density lipoprotein (Supplemental Figure IIA through
IIC), and this elevation was suppressed by siRNA NRF2
(Figure 2C). Similarly, induction of ATF4 mRNA by
electrophilic isoprostane also depended on NRF2 (Figure
2C).

Expression of ATF4 Protein in Response to
OxPAPC Is Mediated by Enhancement of Both
Transcription and Translation
In addition to the inhibition of OxPAPC-induced ATF4
mRNA elevation, silencing of NRF2 resulted in decreased
levels of ATF4 protein (Figure 3A and 3B). NRF2 knock-
down did not influence accumulation of ATF4 protein
induced by classic UPR agonist tunicamycin and did not reduce
phosphorylation of eIF2α because classic UPR agonist tunicamycin and did not reduce
phosphorylation of eIF2α because

Because OxPLs induced phosphorylation of eIF2α (Gar-
galovic et al14 and Figure 3A), we further tested the role of
the PERK-eIF2α mechanism in OxPAPC-induced elevation of
ATF4 protein. Knockdown of PERK using siRNA resulted in
a 74%±17% decrease of PERK mRNA. Silencing of PERK
induced no change in ATF4 mRNA (Figure 3C), but it
inhibited elevation of ATF4 protein (Figure 3D) and its
downstream gene VEGF in response to OxPAPC (Figure 3C).
These data support the notion that OxPL-induced elevation of
ATF4 protein (and its downstream genes) involves 2 parallel
mechanisms: NRF2-dependent elevation of ATF4 mRNA and
concomitant enhanced translation of this mRNA because of
PERK-dependent phosphorylation of eIF2α.

Figure 1. OxPLs upregulate ATF4 mRNA.
A, Kinetics of ATF4 mRNA elevation
induced by OxPAPC. HUVECs were stimu-
lated in quadruplicate with 130 µmol/L
OxPAPC for the indicated time periods. The
number of experiments was increased by
the addition of Trizol. Levels of ATF4 mRNA were mea-
sured by quantitative real-time polymerase
chain reaction (qRT-PCR) and normalized to
the β2-microglobulin mRNA levels. The
figure shows only mean values. *P<0.05,
**P<0.01, and ***P<0.001 versus time-
matched controls. B, Concentration depen-
dence of ATF4 mRNA elevation in HUVECs
treated with different classes of OxPLs.
OxPLs were generated by oxidation of
phospholipid precursors containing sn-1-
palmitic and sn-2-arachidonic acids and the
following head groups: phosphocholine
(OxPAPC), phosphoserine (OxPAPS), phos-
phoglycerol (OxPAPE), phosphoethano-
lamine (OxPAPE) or phosphat (P2XPAPE).
Cells were treated with OxPLs at the indi-
cated concentrations for 4 hours. Thereaf-
ter, mRNA from combined quadruplicates
was analyzed as described in A. Asterisks
indicate statistically significant positive cor-
relation between OxPL concentrations and
ATF4 mRNA levels (P<0.05, **P<0.01,
***P<0.001). C, Concentration dependence
of ATF4 mRNA elevation in human carotid
artery (HCAECs), human aortic (HAECs),
and human uterine microvascular
endothelial cells treated with
different molecular species of phosphatidylcholine containing sn-1-
and the following oxidized sn-2 residues: azelaic acid (PAzPC),
5,6-epoxy isoprostane E2 (PEIPC), glutaric acid (PGPC), (5-oxo)valeric acid (POVPC), monohydroperoxide (PAPC-OOH) and monohydroxide
(PAPC-OH). Stimulation and analysis were performed as described in B.
NRF2 Binds to a Site in the ATF4 Promoter Region

To characterize the mechanisms of NRF2-dependent elevation of ATF4 mRNA, we performed in silico analysis of the ATF4 gene 5’-upstream region and found a putative ARE site at position -290/280. Chromatin immunoprecipitation demonstrated OxPAPC-induced binding of NRF2 protein to a DNA region (-363/-183) containing this site (Figure 4A). Lack of NRF2 binding to a neighboring promoter region (-201/-19; data not shown) and absence of DNA amplification in samples precipitated by nonimmune IgG (Figure 4A) validate binding specificity. Amplification of a fragment of GCLM gene immunoprecipitated with anti-NRF2 from HUVECs treated with electrophilic compound sulforaphane (Figure 4A) served as the positive control.

The ELISA-based NRF2 assay demonstrated significantly higher binding of NRF2 to consensus ARE in nuclear extracts prepared from OxPAPC-treated HUVECs compared with unstimulated cells (3.17±0.57 fold, P=0.0042). To further characterize binding of NRF2 to the putative ARE within ATF4 promoter, wild-type and mutated oligonucleotides representing this ARE were synthesized. The wild-type oligonucleotide effectively competed with binding of NRF2 to immobilized consensus ARE (Figure 4B and 4C). Single substitution of G to T strongly inhibited the ability of ATF4 ARE to compete with NRF2 binding to immobilized consensus ARE (Figure 4B and 4C). Taken together with chromatin immunoprecipitation data, these results allow the hypothesis to be made that NRF2 induced by OxPAPC binds to a regulatory ARE site in the ATF4 promoter and stimulates transcription of this gene.

NRF2 Is Important for Angiogenic Effects of OxPLs

Finally, we obtained evidence that the NRF2 pathway is functionally important for the angiogenic switch induced by OxPLs. NRF2 knockdown inhibited both OxPAPC-induced expression of the ATF4 downstream gene VEGF and HUVEC sprouting (Figure 5A and 5B). Incomplete inhibition of sprouting may be because, apart from VEGF, angiogenic effects of OxPLs are mediated by additional autocrine mediators, such as COX-2-derived prostanoids. In support of this possibility, we found that induction of COX-2 by OxPAPC is independent of NRF2 (Supplemental Figure IV).

Discussion

The major finding done in this work is a novel mechanism of cross-talk between cellular stress signaling pathways. OxPLs are known to induce both UPR and ESR pathways. Previous studies demonstrated elevated phosphorylation of eIF2α in response to OxPAPC, suggesting that selective translation

Figure 2. Upregulation of ATF4 mRNA by OxPAPC depends on NRF2. A, Electrophilic properties are important for elevation of ATF4 mRNA. Reduction by sodium borohydride of electrophilic groups in OxPAPC (130 μmol/L, 6 hours) inhibited elevation of ATF4 mRNA, whereas the electrophilic compounds 8-iso-PGJ2 (50 μmol/L, 6 hours), 5-deoxy-A12,14-PGJ2 (50 μmol/L, 6 hours), and butylated hydroxyanisole (BHA, 200 μmol/L) elevated ATF4 mRNA levels. B, siRNA against NRF2 inhibits elevation of ATF4 and GCLM mRNA in HUVECs treated with OxPAPC (130 μmol/L, 6 hours). Efficiency of NRF2 knockdown, determined by quantitative real-time polymerase chain reaction (qRT-PCR), was 70%±7%. C, siRNA against NRF2 inhibits elevation of ATF4 mRNA in HUVECs treated with oxidized-low-density lipoprotein (100 μg/mL, 6 hours) or 8-iso-PGJ2 (50 μmol/L, 6 hours). After mRNA isolation, expression of ATF4 mRNA was quantified by qRT-PCR and normalized to β2-microglobulin mRNA. Efficiency of NRF2 knockdown, determined by qRT-PCR, was 85%±12%.

Figure 2 (Continued).
duced by this phosphorylation plays a significant role in upregulation of ATF4 protein in cells treated with OxPLs. Here we show that in parallel with enhanced phosphorylation of eIF2α, OxPLs upregulate ATF4 mRNA levels via NRF2-dependent mechanisms. Therefore, the convergence of selective translation of ATF4 with NRF2-dependent elevation of ATF4 mRNA may amplify effects of OxPAPC on expression of ATF4 protein and its downstream targets, such as VEGF. Furthermore, ATF4 and NRF2 can act as direct coactivators of the transcription of genes such as heme oxygenase-1, which because of its angiogenic properties may have an additional impact on OxPL-induced angiogenesis.

The existence of points of convergence between electrophilic and unfolded protein stress pathways strongly suggests that optimal adaptation of cells to environmental and endogenous stresses requires tight coordination of ESR and UPR reactions. Several groups showed that combined concentrations of OxPLs in human and animal hypercholesterolemic plasma, as well as in atherosclerotic vessels, can reach levels of tens of micromoles per liter or kilogram of wet weight. Thus, at least in pathologies such as hypercholesterolemia and atherosclerosis, combined concentrations of active OxPL species achieve the levels that upregulated ATF4 and VEGF in our experiments. Moreover, our data demonstrate that NRF2-dependent upregulation of ATF4 mRNA is not unique for OxPLs but is also induced by other oxidized lipids relevant for cardiovascular pathology, including oxidized low-density lipoprotein and electrophilic (iso)prostaglandins.

In the context of biological importance, our findings are likely to have implications for vascular biology. The ATF4 branch of UPR is increasingly recognized as a pathogenic mechanism in vascular damage characteristic of diabetes and atherosclerosis. ATF4 has been shown to play a role in induction of VEGF and angiogenic reactions associated with diabetic retinopathy, destabilization of lesions, and response to balloon injury. Furthermore, ATF4 is upregulated by oxidative stress and cardiovascular disease risk factors, including cholesterol, homocysteine, oxidized low-density lipoprotein, and OxPLs. Enhanced levels of ATF4 and its downstream target proteins were documented in atherosclerotic vessels, where ATF4-induced genes can execute apoptosis of macrophages thus increasing vulnerability of atheroma. These data characterize ATF4 as an important player in cardiovascular disease. Although NRF2 is usually regarded as an atheroprotective factor, a gene knockout study has demonstrated amelioration of atherosclerosis in Nrf2-null mice. Our data allow it to be hypothesized that in addition to upregulation of scavenger receptors, proatherogenic action of NRF2 may be explained by its ability to induce ATF4 and its downstream product VEGF, which, among other activities, is capable of recruiting monocytes and stimulating plaque formation. Furthermore, it has been shown that regulation of a large proportion of UPR genes,
including ones that are ATF4 regulated, in mouse liver and intestine depends on intact Nrf2.34 Our data offer a potential mechanism of this effect and allow the hypothesis that in addition to vascular wall cells, the NRF2-ATF4 positive cross-talk may be important in other tissues, organs, and disease states.

Figure 4. NRF2 binds to an ARE site in ATF4 promoter. A, Chromatin immunoprecipitation using anti-NRF2 and control IgG. After 2 hours of stimulation with 130 μmol/L OxPAPC or 5 μg/mL sulforaphane or vehicle, DNA-protein crosslinking was performed using 1% formaldehyde. Immunoprecipitated DNA fragments containing ARE sites from the ATF4 and GCLM genes were amplified by polymerase chain reaction, separated in polyacrylamide gel, and visualized by ethidium bromide staining. B and C, NRF2/ARE binding competition assay. Binding of

Figure 4 (Continued). NRF2 from control and OxPAPC-stimulated HUVEC nuclear extracts to immobilized consensus ARE site was quantified using the ELISA-based TransAM NRF2 kit and is expressed as optical density at 450 nm. Two micrograms of nuclear extracts was incubated with the indicated amounts of double-stranded nucleotides corresponding to the ARE consensus binding site, putative ARE site from ATF4 promoter, or its mutated version containing a single-nucleotide mutation.

Figure 5. Knockdown of NRF2 inhibits OxPAPC-induced angiogenic switch. A, VEGF mRNA was quantified in control- and NRF2 siRNA-transfected HUVECs treated with OxPAPC (130 μmol/L, 6 hours). Stimulation was terminated by the addition of Trizol. VEGF mRNA expression was quantified by quantitative real-time polymerase chain reaction (qRT-PCR) and normalized to β2-microglobulin mRNA levels. Efficiency of NRF2 knockdown, determined by qRT-PCR, was 70%±7%. B, Knockdown of NRF2 inhibits formation of sprouts by HUVECs in response to OxPAPC. Forty-eight hours after cell transfection with control or NRF2 siRNA, cell spheroids were formed and embedded in fibrin gels. Thereafter, spheroids were incubated for 24 hours in the presence of medium 199 containing 130 μmol/L OxPAPC and then stained with tetramethyl rhodamine isothiocyanate-conjugated phalloidin for the sprout length measurement.
In summary, the data presented in this report support the notion that OxPLs induce an angiogenic switch in endothelial cells at least partially acting via a cross-talk between NRTF2 and ATF4, thus leading to induction of VEGF. The involvement of NRTF2 in regulation of ATF4 and VEGF by atherogenic lipids opens new possibilities for specific interventions.

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Disclosures
None.

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Supplement material

Experimental procedures

Materials- 8-iso prostaglandin A₂ (8-iso-PGA2) and 15-deoxy-Δ12,14-prostaglandin J₂ (15-deoxy-Δ12,14-PGJ2) were purchased from Cayman Chemical. Butylated hydroxyanisol (BHA), tert-butylhydroquinone (tBHQ) and sulforaphane (Sul) were from Sigma-Aldrich. NRF2 and ATF4 antibodies were from Santa Cruz Biotechnology, anti-actin from DakoCytomation. Antibodies to total eIF2α and phosphorylated form were purchased from Cell Signaling Technology.

Cell culture- Endothelial cells were purchased from Lonza (HUVECs, HAECs and HCAECs) and from Technoclone (HUMECs). Cells were grown at 37°C and 5% CO₂ in medium 199 containing 20% fetal calf serum (FCS), 1 U/mL heparin, 50 µg/mL bovine EC growth supplement (Technoclone), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were used up to passage 5. HUVECs were stimulated with lipids and other agonists in medium M199 containing 2% FCS.

Lipids- Synthetic 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphate (PAPA), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoserine (PAPS), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoglycerol (PAPG), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine (PAPE), and 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PAzPC) were purchased from Avanti Polar Lipids. Phospholipids (PLs) were oxidized by air exposure of a thin lipid film. The oxidation was stopped when approximately 80% of the starting PL was oxidized. Oxidation was monitored by thin-layer chromatography and electrospray ionization-mass spectrometry. 1-Palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) were synthesized as described previously. 1-Palmitoyl-2-(5,6-epoxy isoprostane E2)-sn-glycero-3-phosphocholine (PEIPC)-enriched fraction was purified from OxPAPC as described. A mixture of 1-palmitoyl-2-(12-hydroperoxy-5,8,10,14-eicosatetraenoyl)-sn-glycero-3-phosphocholine and 1-palmitoyl-2-(15-hydroperoxy-5,8,11,13-eicosatetraenoyl)-sn-glycero-3-phosphocholine was synthesized by an enzyme-catalyzed reaction from PAPC using soybean lipoxygenase (type I-B, Sigma-Aldrich) in the presence of deoxycholate. Reduction of crude mixture containing phospholipid hydroperoxides with triphenylphosphine producing 1-palmitoyl-2-(12-hydroxy-5,8,10,14-eicosatetraenoyl)-sn-glycero-3-phosphocholine and 1-palmitoyl-2-(15-hydroxy-5,8,11,13-eicosatetraenoyl)-sn-glycero-3-phosphocholine (PAPC-OOH) was performed as described. Purification of PC-O(O)H was performed by column chromatography on silica gel 60 (70-230 mesh, 60A, Sigma-Aldrich). The lipids were eluted with a gradient of chloroform-methanol-water from 100:0:0 to 65:25:4 (v/v/v) followed by solid-phase
extraction on Supelclean LC18 columns (1 g, Supelco) using a gradient from 50% to 100% methanol in water. Fractions were analysed by TLC using a developing system chloroform-methanol-water (100:50:10, v/v/v) with subsequent staining with a 10% copper sulphate solution in 8.5% orthophosphoric acid and heating to 120°C. Fractions containing compounds of interest were concentrated on a rotary evaporator under vacuum. MS-analysis confirmed the purity of PL-O(O)H. Reduction of electrophilic groups present in OxPAPC by sodium borohydride was performed as described. Concentration of phospholipids was determined by phosphorus assay.

The dried lipids were resuspended by vigorous vortexing in medium 199 supplemented with 2% FCS prior to use in cell culture experiments.

**RNA isolation, cDNA synthesis and quantitative PCR** - Isolation of RNA from HUVECs was performed using Trizol reagent (Invitrogen). GeneAmp RNA-PCR kit and oligo d(T)16 primers (Applied Biosystems) were used for cDNA synthesis from 900 ng of total RNA. Quantitative real-time PCR was performed using LightCycler instrument (Roche Diagnostics) and FastStart SYBR Green Master Mix (Roche Diagnostics). Sequences of primers are available on request.

**Western blotting** - After lysis of cells in Laemmli buffer protein samples were separated on SDS-polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore) by electroblotting. Membranes were probed with rabbit antibodies against ATF4, NRF2, phospho-eIF2α, total eIF2α and β-actin. Anti-rabbit IgG conjugated with peroxidase and the LumiGLO chemiluminescent substrate (Cell Signaling Technology) were used for detection of bound primary antibodies. FluorChem HD2 imager (Alpha Innotech) was used for chemiluminescent detection.

**RNA interference** - NRF2-, PERK-specific and control siRNAs were purchased from Ambion and Qiagen. Cells were transfected with 100 nmol/L of siRNA using PEI reagent in serum- and antibiotic-free medium 199 for 4 hours. Thereafter the medium was changed to medium 199 containing 20% FCS and cells were grown for 48 hours. Efficiency of silencing was checked at the mRNA and/or protein levels.

**Chromatin immunoprecipitation (ChIP)** - ChIP analysis was performed using EZ-Chip kit (Upstate). HUVECs were treated with 130 µmol/L of OxPAPC or 5 mmol/L of sulforaphane in medium 199 containing 2% FCS. After 2 hours, formaldehyde was added to the final concentration of 1%. Cross-linking was stopped after 8 minutes of incubation at room temperature by addition of glycine to the final concentration of 0.125 mol/L. Cell extracts were sonicated using Bandelin sonicator by 4 bursts of 10 seconds at 30% power. Amounts of extracts corresponding to 1x10⁷ cells were incubated overnight with antibodies against ATF4 or non-immune rabbit IgG. Before immunoprecipitation, one percent of extract volume was removed and served as an input. DNA fragments from immunoprecipitated complexes and inputs were released by heating at 65°C.
overnight and were purified according to the vendor’s protocol. Purified immunoprecipitated and input DNA was analyzed by PCR. Following primers were used: for ATF4 promoter region containing putative ARE (-290/-280), 5´-CCTCGGCCTTCACAATAAAA and 5´- TTATGGCCTCACGAAAGGAG; for GCLM ARE, 5´- CGCGGGATGAGTAACGGT and 5´- GGAGAGCTGATTCCAAACTGA. PCR products (181 and 101 bps, respectively) were resolved in polyacrylamide gels and stained with ethidium bromide.

**NRF2 binding competition assay** – NRF2-DNA interactions were additionally characterized using ELISA - based TransAM NRF2 Kit (Active Motif). The procedure is based on detection of NRF2 bound to consensus ARE immobilized in 96-well plate. Nuclear extracts from OxPAPC-stimulated and untreated HUVECs were prepared using NE-Per Kit (Pierce) after 2 hours of incubation. 2 µg of nuclear extract protein were incubated in the plate containing immobilized consensus ARE either in the presence or absence of competing oligonucleotide for 1 hour. The wells were washed three times, and bound NRF2 was detected by NRF2 antibody and secondary antibody conjugated with horseradish peroxidase. The signal was detected spectrofotometrically at 450 nm. Sequences of oligonucleotides were the following: 5´- GTCACAGTGACTAGCAGAATCTG (consensus ARE), 5´- CGCCCACTGCGCTGACACCGGAAGCGAGGC (wild-type putative ARE from ATF4 promoter) and 5´- CGCCCACTGCGCTACACCGGAAGCGAGGC (mutated ATF4 ARE; mutated nucleotide is in bold), respectively.

**Spheroid assay of angiogenesis in vitro**- 48 hours after transfection with either control or NRF2 siRNAs cell spheroids were formed and embedded in fibrin gels as described. Polymerized gels were overlain with medium 199 supplemented with 2% FBS without or with inclusion of OxPAPC (130 µmol/L). After 24 hours gels were fixed and actin structures were visualized with TRITC-conjugated phalloidin (Sigma-Aldrich). AnalySIS software (Soft Imaging System GmbH) was used for determination of the length and number of sprouts per spheroid. For each experimental conditions data from at least 20 spheroids was used.

**Supplemental Figures legends**

**Supplementary Figure I.** siRNA against NRF2 inhibits OxPAPC induced elevation of GCLM mRNA. HUVECs were treated with OxPAPC (130 µmol/L) for 6 hours). After mRNA isolation, level of mRNA was quantified by RT-qPCR and normalized to the ß2-microglobulin mRNA level. Efficiency of NRF2 knockdown determined by qRT-PCR was 70 ± 7%.
Supplementary Figure II. Effect of native and oxidatively modified LDL on the level of ATF4, GCLM and GCLC mRNAs (A, B and C respectively). HUVECs were treated with LDL or OxLDL (100 µg/ml, 6 hours) after mRNA isolation, levels of mRNAs were quantified by RT-qPCR and normalized to the β2-microglobulin mRNA level.

Supplementary Figure III. siRNA against NRF2 inhibits elevation of ATF4 mRNA in HUVECs treated with OxPAPC (130 µmol/L, 6 hours). Effect of normalization to mRNA of different housekeeping genes. After mRNA isolation, expression of ATF4 mRNA was quantified by RT-qPCR and normalized to the β2-microglobulin mRNA (A), GAPDH mRNA (B) or PBGD mRNA (C).

Supplementary Figure IV. Effect of siRNA-mediated knockdown of NRF2 on the induction of COX-2 by OxPAPC. siRNA-transfected HUVECs were treated with OxPAPC (130 µmol/L, 6 hours). Level of mRNA as quantified by RT-qPCR and normalized to β2-microglobulin mRNA level.

References


Supplementary Figure I

GCLM, mRNA, fold of control

- No lipid
- OxPAPC

Control siRNA
NRF2 siRNA

P<0.05
Supplementary Figure II

A

ATF4 mRNA, fold of control

Control LDL OXLDL

P<0.01

B

GCLM mRNA, fold of control

Control LDL OXLDL

P<0.01

C

GCLC mRNA, fold of control

Control LDL OXLDL

P<0.01
Supplementary Figure III

A

ATF4 mRNA, fold of control normalized to β2M mRNA

Control siRNA    NRF2 siRNA

No lipid         OxPAPC

P < 0.01

B

ATF4 mRNA, fold of control normalized to GAPDH mRNA

Control siRNA    NRF2 siRNA

No lipid         OxPAPC

P < 0.05

C

ATF4 mRNA, fold of control normalized to PBGD mRNA

Control siRNA    NRF2 siRNA

No lipid         OxPAPC

P < 0.01
Supplementary Figure IV

COX-2 mRNA, fold of control

- Control siRNA
- NRF2 siRNA

No lipid
OxPAPC

NS