OKL38 is an oxidative stress response gene stimulated by oxidized phospholipids

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Abstract Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC) is present in oxidative modified LDL and accumulates in lesions of many chronic inflammatory diseases, such as atherosclerosis. In a microarray study, OxPAPC has been demonstrated to modulate the expression of >700 genes in human aortic endothelial cells. We found that the levels of mRNA for OKL38 [also named Bone marrow Derived Growth Factor (BDGI)], a tumor growth inhibitor, were strongly increased by OxPAPC. Here, we report that OKL38 is regulated by an oxidative signal induced by OxPAPC and its component lipid 1-palmitoyl-2-epoxyisoprostane E2-*sn*-glycero-3-phosphorylcholine. The stimulation of OKL38 by OxPAPC depends on superoxide production, because the NADPH oxidase (Nox) inhibitor apocynin and the superoxide scavenger *N*-acetyl cysteine block this stimulation. Oxidative stress by *tert*-butylhydroquinone treatment also induced the expression of OKL38. The stimulation of OKL38 expression by OxPAPC is mediated via transcription factor nuclear factor E2-related factor (Nrf2), a common factor involved in the regulation of oxidative stress-stimulated genes. Activation of Nrf2 induces the expression of OKL38, whereas small interfering RNA knockdown of Nrf2 blocks the stimulation of OKL38 by OxPAPC. Our results suggest that OKL38 is regulated via the Nox/Nrf2 pathway in response to oxidative stress stimuli.—Li, R., W. Chen, R. Yanes, S. Lee, and J. A. Berliner. **OKL38 is an oxidative stress response gene stimulated by oxidized phospholipids. *J. Lipid Res.* 2007.** 48: **709–715.**

Supplementary key words oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine • OKL38/BDGI • superoxide • reduced nicotinamide adenine dinucleotide phosphate oxidase • nuclear factor E2-related factor • atherosclerosis

Oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (OxPAPC) was identified as a major bioactive component of mildly modified LDL (1). It is present in atherosclerotic lesions and in the sites of other chronic inflammatory diseases (2–4). Application of OxPAPC triggers inflammatory responses in vitro and in vivo via increased expression of inflammatory genes and the activation of monocyte binding to endothelial cells (4–7). In a microarray study, OxPAPC was demonstrated to regulate the expression of >700 genes in human aortic endothelial cells (HAECs) (8). Among the genes stimulated by OxPAPC are inflammatory genes such as interleukin-8 and Monocyte Chematoactic Protein-1 (MCP-1), sterol metabolism genes such as LDL receptor, unfolded protein response genes such as Activating Transcription Factor-3 (ATF3), and redox genes such as heme oxygenase-1 (HO-1). This study focuses on the regulation of OKL38, one of the most highly induced genes in response to OxPAPC.

OKL38 was identified as a tumor suppressor gene that inhibits tumor cell growth (9, 10). Its expression was lost or reduced in tumor cell lines and tumor tissues (10, 11). The only study on OKL38 regulation showed that administration of human chorionic gondotropin to rats stimulated the expression of OKL38 in mammary gland and ovary but not in other tissues (12). However, little is known about the mechanism of OKL38 regulation and its molecular function.

We and others have shown that OxPAPC induces superoxide production in endothelial cells and that superoxide induction by OxPAPC is mediated by NADPH oxidase (Nox) and uncoupled endothelial Nitric Oxide Synthase (eNOS) (13, 14). In cells treated with OxPAPC, superoxide was shown to be involved in the expression of interleukin-8 in HAECs and of Matrix Metalloprotease-2 (MMP2) in bovine aortic endothelial cells (13, 14).

Oxidative stress is a condition in which levels of intracellular reactive oxygen species (ROS) exceed the antioxidant capacity of the cells. ROS, in which superoxide is the primary species, are involved in regulating multiple biological processes. ROS not only cause oxidative damage to biomolecules such as DNA, protein, and lipids but also regulate the expression of redox-sensitive genes (15, 16).

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Abbreviations: ARE, antioxidant response element; HAEC, human aortic endothelial cell; HO-1, heme oxygenase-1; NAC, *N*-acetyl cysteine; Nox, NADPH oxidase; Nrf2, nuclear factor E2-related factor; OxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine; PEIPC, 1-palmitoyl-2-epoxyisoprostane E2-*sn*-glycero-3-phosphorylcholine; PGPC, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine; POVPC, 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphorylcholine; ROS, reactive oxygen species; siRNA, small interfering RNA; tBHQ, *tert*-butylhydroquinone.

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Nuclear factor E2-related factor (Nrf2) has been shown to be a major transcription factor that mediates ROS- and oxidative stress-induced gene expression (17). Studies with Nrf2-deficient cells have demonstrated the importance of Nrf2 in the expression of detoxifying and antioxidant enzymes in response to oxidative stress (18). For example, the stimulation of the antioxidant enzyme HO-1 requires Nrf2 (19). HO-1 is among the most strongly stimulated genes by OxPAPC (8). In macrophages, it was shown that OxPAPC activates the HO-1 promoter. The HO-1 promoter contains the antioxidant response element (ARE) that binds to Nrf2 (20). This suggests that Nrf2 is a mediator of the OxPAPC regulation of redox genes such as HO-1. In this study, we examine the regulation of OKL38 by OxPAPC and the role of oxidative signal and Nrf2. We report that OxPAPC stimulates the expression of OKL38 in endothelial cells, monocytes, and macrophages and that the stimulation of OKL38 expression requires the activation of Nox and Nrf2.

EXPERIMENTAL PROCEDURES

Materials

Cell culture media and reagents were obtained from Irvine Scientific, Inc., and Invitrogen, Inc. FBS was obtained from Hyclone, Inc. PAPC and 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphorylcholine (POVPC) were purchased from Avanti Polar Lipids, Inc. OxPAPC and 1-palmitoyl-2-epoxyisoprostane E2-*sn*-glycero-3-phosphorylcholine (PEIPC) were prepared and analyzed in our laboratory as described previously (21). 1-Palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine (PGPC) was purchased from Cayman, Inc. Apocynin, Rac1 inhibitor (catalog No. 553508), and *N*-acetyl cysteine (NAC) were purchased from Calbiochem. Protease inhibitor cocktail was purchased from Sigma, Inc. Antibody against Nrf2 was obtained from Santa Cruz Biotechnology. HRP-conjugated secondary antibodies were obtained from Cell Signaling, Inc. Scrambled control small interfering RNA (siRNA) was obtained from Invitrogen. siRNA of Nrf2 (Hs_NFE2L2_4 HP) and HiPerFect® were obtained from Qiagen, Inc. The Nrf2 expression plasmid used was pSPORT6-mNrf2 and was purchased from Openbiosytems, Inc.

Cell culture and treatment

HAECs were prepared and cultured as described previously (22). Human monocytes were isolated and differentiated into macrophages in vitro as described previously (23). In most cases, HAECs were treated with OxPAPC for 4 h in M199/0.2% FBS. For the treatment of HAECs with *tert*-butylhydroquinone (tBHQ), serum-free M199 was used. In studies with inhibitors, HAECs were pretreated with the indicated concentrations of inhibitors for 1 h before cotreatment with OxPAPC and inhibitors.

RT-PCR and semiquantitative real-time PCR

Total RNA was isolated with an RNeasy® mini kit from Qiagen according to the manufacturer's instructions. Potential genomic DNA contamination was removed with on-column DNase I digestion. Total RNA (0.5–1 μg) was reverse-transcribed with Bio-Rad's iScript cDNA synthesis kit. The expression of OKL38 was measured at the mRNA level using semiquantitative real-time PCR essentially as described previously (24). The same experiment was repeated three or more times. Primers used to measure OKL38 and Nrf2 expression were as follows: for OKL38: forward, TCCTCTACGCCCGCCACTACAACATCC; reverse, GGTCCTGGAACACGGCCTGGCAGTCTTC; for Nrf2: forward, AGCATGCCCTCACCTGCTACTTTA; reverse, ACTGAGTGTTCTGGTGATGCCACA. The expression of target genes was calculated as fold increase relative to controls and normalized to GAPDH.

Cell lysates, nuclear extract, and Western blot

For whole cell lysates, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, and 0.5% sodium deoxycholate, supplemented with protease inhibitor cocktail and 1 mM PMSF) at 4°C for 30 min. After centrifugation for 5 min at 4°C, supernatant was collected as whole cell lysates. Nuclear extract was prepared according to Osborn, Kunkel, and Nabel (25) with the modification that cells washed with cold PBS were suspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, and 0.1% Nonidet P-40, plus freshly prepared protease inhibitor cocktail and 1 mM PMSF). After 10 min of incubation on ice, the suspension was centrifuged at 10,000 *g* for 5 min at 4°C. The supernatant was collected as cytosolic extract. The pellet was resuspended in a proper volume of buffer B (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 0.42 M NaCl, 0.5 mM EDTA, and 1 mM DTT, with protease inhibitor cocktail and 1 mM PMSF added immediately before use) and put on ice for 10 min. After centrifugation at 12,000 *g* for 10 min at 4°C, the supernatant was collected as nuclear extract. Protein concentration was determined with a Bio-Rad DC protein assay kit. SDS-PAGE and Western blotting were essentially performed as described (26). Lysates and extracts were run on a 4–12% gradient SDS-PAGE gel. The proteins were then transferred to a polyvinylidene difluoride membrane and blotted with the indicated primary and secondary antibodies. Signal was developed with ECL™-Plus (GE Healthcare) and recorded with VersaDoc™ (Bio-Rad, Inc.).

siRNA transfection in HAECs

HAECs were trypsinized 1 day before transfection and plated at 12,500 cells/cm^2. The next day, cells were washed with and cultured in M199/10% FBS before transfection. siRNA was transfected into HAECs with HiPerFect® using 5–20 nM siRNA and 10–15 μl of HiPerFect® lipid for each well of a six-well plate. The next day, the medium was replaced with regular culture medium. At 48 h after transfection, the cells were used for analysis of gene expression or for treatment.

Nitroblue tetrazolium assay

A modification of the method of Banfi et al. (27) was used to measure intracellular levels of superoxide. HAECs were plated on a 96-well plate and grown to confluence. The cells were washed with serum-free M199 medium and then treated with various reagents in serum-free medium containing 0.2 mg/ml nitroblue tetrazolium. After 1 h of treatment, the medium was removed. The cells were then fixed, and the extracellular nitroblue tetrazolium was removed with methanol. After adding the proper amounts of 2 M KOH and DMSO, relative superoxide production was measured as optical density at 700 nm.

RESULTS

OxPAPC and its component lipid PEIPC stimulate the expression of OKL38

From the microarray study, it appeared that OKL38 was one of the most strongly stimulated genes by OxPAPC. To confirm the regulation of OKL38 by OxPAPC, HAECs were treated with OxPAPC and the expression of OKL38 was analyzed by real-time PCR (**Fig. 1A**). OxPAPC at 5 μg/ml increased OKL38 expression by ~5-fold. The stimulation

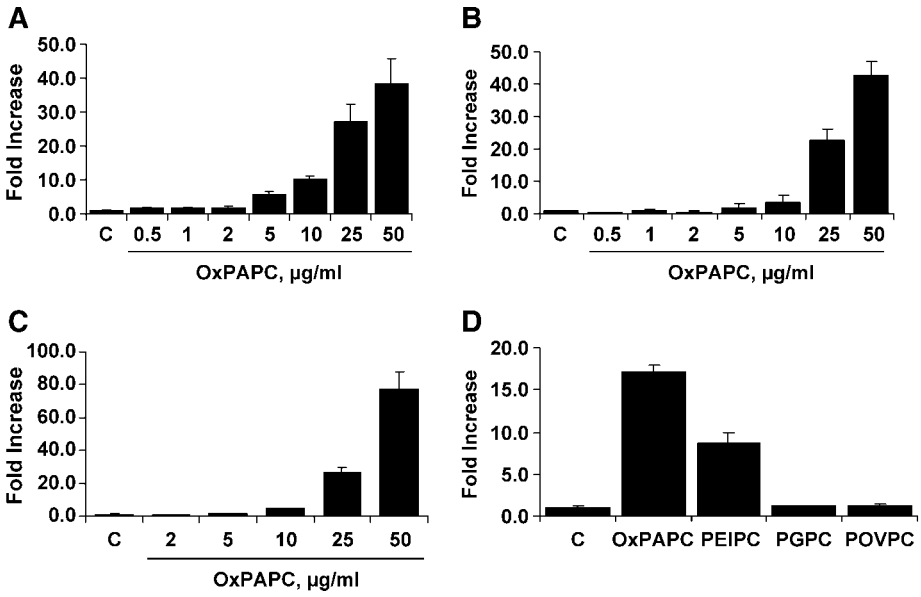

Fig. 1. Oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (OxPAPC) and its component lipid 1-palmitoyl-2-epoxyisoprostane E2-*sn*-glycero-3-phosphorylcholine (PEIPC) stimulate the expression of OKL38. A–C: Human aortic endothelial cells (HAECs) (A), THP-1 cells (B), and human peripheral macrophages (C) were treated with different concentrations of OxPAPC for 4 h in M199/0.2% FBS (HAECs) or RPMI-1640/0.2% FBS (THP-1 cells and macrophages). RNAs were isolated from the treated cells. The expression level of OKL38 was measured by real-time PCR. D: HAECs were treated with OxPAPC (50 μg/ml), PEIPC (enriched fraction; 5 μg/ml), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphorylcholine (PGPC; 50 μg/ml), and 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phosphorylcholine (POVPC; 50 μg/ml) for 4 h. OKL38 expression level was analyzed. C, control. Error bars represent SD.

of OKL38 increased with the concentration of OxPAPC and reached >30-fold with treatment by 50 μg/ml OxPAPC. A similar dose response was observed in monocytic THP-1 cells (Fig. 1B) and primary human macrophages (Fig. 1C). Among the three major bioactive components of OxPAPC (28), only PEIPC, but not POVPC or PGPC, stimulated the expression of OKL38 (Fig. 1D).

Nox is involved in the stimulation of OKL38 by OxPAPC

OxPAPC regulates gene expression via several signal transduction pathways (8, 26, 29, 30). To examine the mechanism of OKL38 stimulation by OxPAPC, we tested the effect of a number of signal pathway inhibitors on OKL38 stimulation by OxPAPC. The inhibitors used included the protein kinase A inhibitor H89, the protein kinase C inhibitor bisindolylmaleimide I, the Extracellular signal-regulated protein kinase (Erk) kinase inhibitor PD98059, the mitogen-activated protein p38 kinase inhibitor SB203580, and the phosphatidylinositol 3-kinase inhibitor LY294002. We found that apocynin, an inhibitor of Nox, dramatically blocked OKL38 stimulation by OxPAPC (**Fig. 2A**). SB203580 also partially inhibited OKL38 stimulation by high concentrations of OxPAPC, whereas other inhibitors had no effect (data not shown).

Nox is an enzyme with multiple subunits. One of the subunits is Rac1, which is involved in the activation of Nox (31). Consistent with the apocynin results, Rac1 inhibitor completely inhibited the stimulation of OKL38 by OxPAPC (Fig. 2B).

OKL38 is an oxidative stress-regulated gene

The effect of Nox inhibitor suggests that OKL38 may be an oxidative stress response gene. Consistent with oxidative regulation, NAC, a scavenger of superoxide, can totally block the stimulation of OKL38 by OxPAPC (**Fig. 3A**). Additional results using tBHQ also support a role of oxidative stress in OKL38 regulation. tBHQ is a phenolic antioxidant that can undergo autooxidation to form redox-active semiquinone anion radicals that put oxidative stress on cells (32). As shown in Fig. 3B, tBHQ dose-dependently increased superoxide production in HAECs, as measured by nitroblue tetrazolium assay. Importantly, tBHQ, like OxPAPC, also stimulated OKL38 expression (Fig. 3C).

OxPAPC and its most active component lipid PEIPC activate Nrf2

Nrf2 is a redox-sensitive transcription factor that mediates oxidative stress-stimulated gene expression. Under normal conditions, Nrf2 is sequestered in the cytoplasm as an inactive complex with its cytosolic repressor Keap1. Upon activation, Nrf2 is released from Keap1, translocated to the nucleus, and activates gene expression (33). Our laboratory has shown that OxPAPC treatment causes Nrf2 translocation to the nucleus in HAECs (N. M. Gharavi, S. P. Gargalovic, I. Chang, J. Araujo, M. J. Clark, A. D. Watson, A. J. Lusis, and J. A. Berliner, unpublished data). Here, we show that among the three major active components of OxPAPC, only PEIPC, but not POVPC or PGPC, causes Nrf2 translocation (**Fig. 4**). This is in parallel

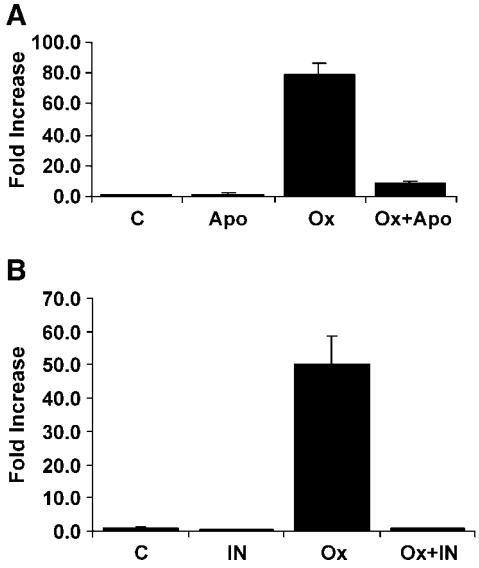

Fig. 2. NADPH oxidase is involved in OKL38 stimulation by OxPAPC. HAECs were treated with or without 1 mM apocynin (A) or 50 μM Rac1 inhibitor (B) for 1 h in M199/0.2% FBS. The cells were then cotreated with inhibitors, with or without 50 μg/ml OxPAPC, for 4 h. The expression of OKL38 was analyzed by real-time PCR. C, control; Ox, OxPAPC; Apo, apocynin; IN, Rac1 inhibitor. Error bars represent SD.

with the result that PEIPC, but not POVPC or PGPC, stimulates OKL38 expression.

Nrf2 accumulation in the nucleus and overexpression stimulate the expression of OKL38

Under normal conditions, Nrf2 undergoes rapid turnover in the cytoplasm via the proteasome pathway (34). Proteasome inhibitors, such as MG115, stabilize Nrf2, resulting in the accumulation of Nrf2 in the nucleus and the activation of target genes (34). Treatment of HAECs with MG115 resulted in the stimulation of OKL38 transcription (**Fig. 5A**). Similarly, overexpression of Nrf2 in HEK293 cells also stimulated the expression of OKL38 (Fig. 5B).

Nrf2 is required for OxPAPC-stimulated OKL38 expression

To determine whether Nrf2 is required for OxPAPC-stimulated OKL38 expression, we used siRNA to knock down the expression of Nrf2. Nrf2 siRNA reduced 95% of Nrf2 mRNA expression (**Fig. 6A**) and significantly reduced Nrf2 protein expression in HAECs (Fig. 6B). OKL38 stimulation by OxPAPC was reduced by 86% in cells treated with Nrf2 siRNA (Fig. 6C). Thus, Nrf2 is necessary for OKL38 stimulation by OxPAPC.

DISCUSSION

In this study, we report that OxPAPC stimulates the expression of OKL38 and that this stimulation is mediated via a Nox/Nrf2 pathway. Several pieces of data reported

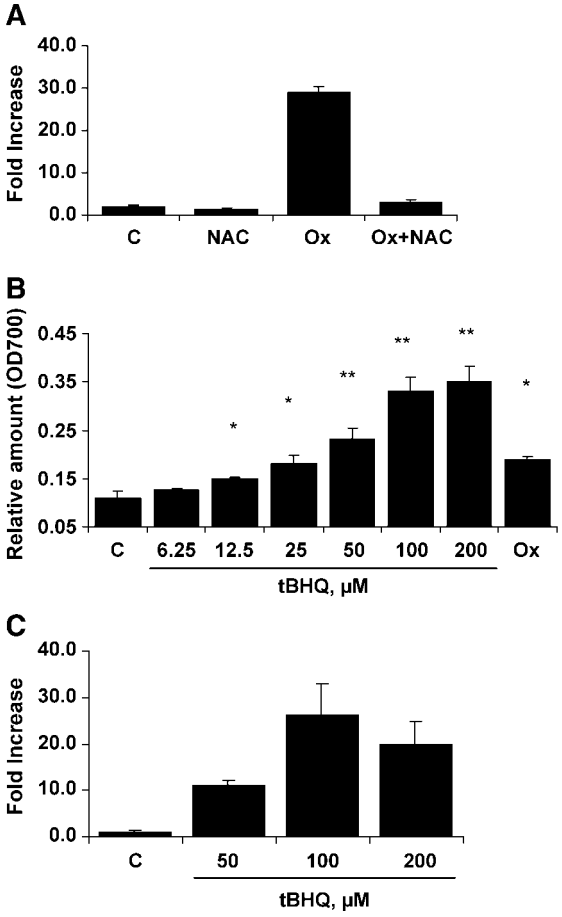

Fig. 3. OKL38 stimulation is a response to reactive oxygen species (superoxide). A: Superoxide scavenger *N*-acetyl cysteine (NAC) blocks OxPAPC-stimulated OKL38 expression. HAECs were pretreated with or without NAC (5 mM) for 1 h in buffered M199/0.2% FBS and then treated with or without 50 μg/ml OxPAPC (Ox) for 4 h. Expression of OKL38 was measured. B: *tert*-Butylhydroquinone (tBHQ) induces intracellular superoxide production in a dose-dependent manner. HAECs were treated with different concentrations of tBHQ for 1 h together with nitroblue tetrazolium in serum-free M199. The intracellular level of superoxide was measured (OD700, for optical density at 700 nm). C: tBHQ induces OKL38 expression. HAECs were treated with tBHQ for 4 h in serum-free M199. The expression of OKL38 was measured. C, Control. * $P < 0.05$, ** $P < 0.01$ (n = 3). Error bars represent SD.

here support the regulation of OKL38 by oxidative stress. First, OKL38 stimulation by OxPAPC requires the activity of Nox, which is a major source of cellular superoxide production in response to OxPAPC (13). Second, scavenging of superoxide by the antioxidant NAC blocks the stimulation of OKL38. Third, oxidative stress induced by tBHQ also stimulates the expression of OKL38.

Nrf2/ARE has been reported to be a major pathway mediating oxidative stress-induced detoxification and antioxidant enzyme expression (17, 18). Previous studies have suggested that OxPAPC stimulates HO-1 via Nrf2 (20). We demonstrate here that the activation of Nrf2 can lead to the induction of OKL38 (Fig. 5) and that knockdown of Nrf2 inhibits OKL38 induction by OxPAPC (Fig. 6C). Gargalovic et al. (35) used a bioinformatics approach to

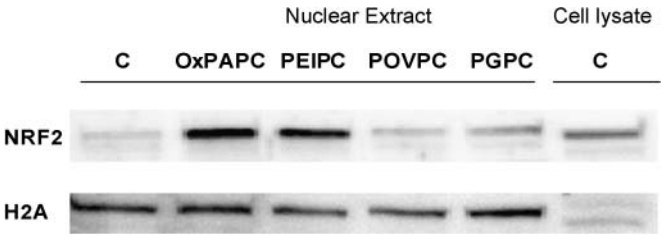

Fig. 4. OxPAPC and its component PEIPC, but not PGPC or POVPC, activate nuclear factor E2-related factor (Nrf2). HAECs were treated with OxPAPC (50 μg/ml), PEIPC (5 μg/ml), PGPC (50 μg/ml), and POVPC (50 μg/ml) for 2 h. Total cell lysates and nuclear extracts were prepared as described. Nrf2 protein translocation to the nucleus was measured by Western blot. C, control.

study the mechanisms involved in OxPAPC-stimulated gene expression. The genes with similar expression patterns in response to OxPAPC were grouped into modules. OKL38 and HO-1 were shown to be in the same module enriched with oxidative stress response genes. Another oxidative stress response gene in this module is glutathione cysteine ligase modifier subunit, which is also activated by Nrf2 (36). Although the promoter of OKL38 has not been clearly identified, sequence analysis of the 5′ end of the OKL38 gene indicates that the two putative OKL38 promoters reported contain ARE-like sequences that could bind Nrf2. These sequences are GCTGACTCACG in putative promoter 1 and CCTGACCTCAGG in putative promoter 2. The detailed mechanism of OKL38 stimulation by OxPAPC via Nrf2 awaits the identification and characterization of the OKL38 promoter.

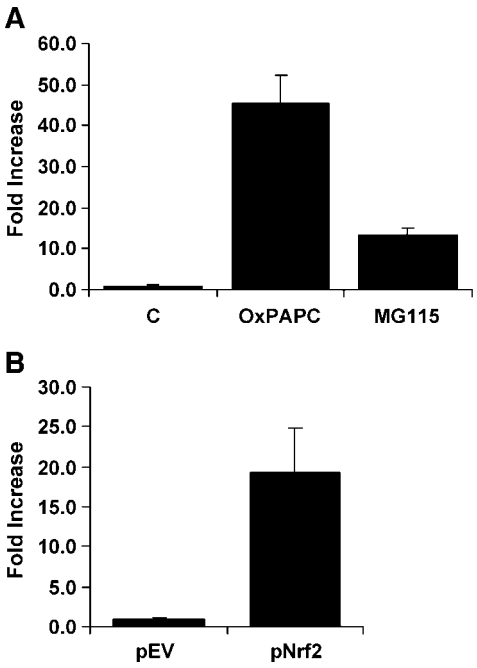

Fig. 5. Increased levels of nuclear Nrf2 and overexpression of Nrf2 induce OKL38 expression. A: MG115 induces OKL38 expression. HAECs were treated with 50 μM MG115 for 4 h, and OKL38 expression was measured. B: Overexpression of Nrf2 stimulates OKL38 expression. HEK293 cells were transfected with empty plasmid (pEV) or plasmid with mouse Nrf2 (pNrf2). At 30 h after transfection, the expression of OKL38 was measured by real-time PCR. C, control. Error bars represent SD.

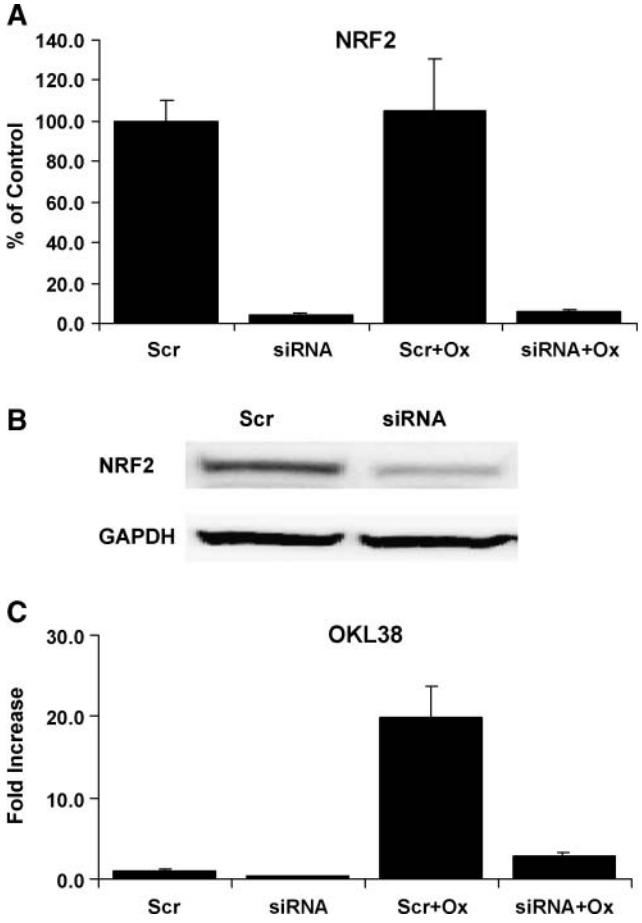

Fig. 6. Nrf2 is required for OxPAPC-stimulated OKL38 expression. A, B: Nrf2 knockdown by Nrf2 small interfering RNA (siRNA). HAECs were transfected with 20 nM siRNA or scrambled RNA control (Scr) for 48 h. Nrf2 mRNA level (A) and protein level (B) were measured by real-time PCR and Western blot, respectively. C: Nrf2 knockdown blocked OKL38 stimulation by OxPAPC. HAECs were transfected with 20 nM siRNA or scrambled RNA. At 48 h after transfection, cells were treated with or without 50 μg/ml OxPAPC (Ox) for 4 h. OKL38 expression was measured. Error bars represent SD.

Oxidative signals play an important role in the pathogenesis of chronic inflammatory diseases such as atherosclerosis by regulating the expression of proatherogenic genes (16). Oxidative signals also regulate the expression of protective antioxidant genes. For example, oxidized LDL and mildly modified LDL stimulate the expression of the glutathione and thioredoxin system genes, including thioredoxin, thioredoxin reductase, and glutathione reductase (37). It has been reported previously that OxPAPC, a component molecule of mildly modified LDL, stimulates the expression of HO-1, an anti-inflammatory and antioxidant gene protecting cells from oxidative stress (38). The data reported here showed that OKL38, like the protective gene HO-1 and Glutamate-Cysteine Ligase, Modifier subunit, is regulated by OxPAPC via a similar

mechanism. It is possible that OKL38 may also protect cells against stress, and its regulation by OxPAPC may represent a feedback protective mechanism.

Data reported in the literature support OKL38 as a tumor suppressor gene. Overexpression of OKL38 inhibits tumor cell growth and induces apoptosis (9, 10). The mechanism underlying this effect is not clear. It has been long appreciated that actively dividing and metabolically hyperactive cells such as tumor cells produce free radicals and ROS (39). One possibility is that OKL38 may respond to oxidative signals induced by hyperproliferation and that OKL38 decreases cell proliferation. In normal cells, OKL38 may inhibit proliferation in cells exposed to oxidative stress, whereas loss of OKL38 would promote tumor growth. Microarray data have shown that OxPAPC can downregulate the expression of cyclins (8). Interestingly, overexpression of OKL38 also inhibits cyclin expression (9). There is the possibility that OKL38 is involved in the downregulation of cyclins by OxPAPC. Another way that OKL38 may protect cells from OxPAPC is through the metabolism of epoxide. This hypothesis is based on the existence of a pyr-redox domain in the C terminus of OKL38 (9, 11). One member of the pyr-redox gene family has been reported to metabolize epoxides (40). It may not be a coincidence that PEIPC, the active component in OxPAPC that stimulates the expression of OKL38, contains an epoxide structure at the *sn*-2 position. It is possible that OKL38 is part of a feedback mechanism to remove the stress that PEIPC puts on cells by metabolizing PEIPC.

In summary, we have demonstrated that oxidized phospholipids stimulate the expression of OKL38 via a Nox/Nrf2 pathway. This result suggests that OKL38 is an oxidative stress response gene that may be associated with chronic inflammatory processes in which OxPAPC accumulates. OKL38 may also be involved in other pathological conditions in which oxidative signals play a role.

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