Increased Transcription of IL-8 in Endothelial Cells Is Differentially Regulated by TNF-α and Oxidized Phospholipids

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Abstract—Oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (Ox-PAPC) upregulates a spectrum of inflammatory cytokines and adhesion molecules different from those induced by classic inflammatory mediators such as tumor necrosis factor-α (TNF-α) or lipopolysaccharide. Interestingly, Ox-PAPC also induces the expression of a set of proteins similar to those induced by TNF-α or lipopolysaccharide, which include the chemokines monocyte chemotactic protein-1 (MCP-1) and interleukin (IL)-8. To elucidate the molecular mechanisms of Ox-PAPC–induced gene expression and to determine whether Ox-PAPC and other inflammatory mediators such as TNF-α utilize common signaling pathways, we examined the transcriptional regulation of IL-8 by Ox-PAPC and TNF-α in human aortic endothelial cells. Both Ox-PAPC and TNF-α induced the expression of IL-8 mRNA in a dose-dependent fashion; however, the kinetics of IL-8 mRNA accumulation between the 2 ligands differed. Ox-PAPC–induced IL-8 mRNA was seen as early as 30 minutes, peaked between 4 and 8 hours, and decreased substantially by 24 hours. In contrast, TNF-α–induced IL-8 mRNA synthesis was elevated at 30 minutes, peaked at 2 hours, and reached basal/undetectable levels by 6 hours. Actinomycin D experiments suggested that both Ox-PAPC and TNF-α regulate the expression of IL-8 at the transcriptional level. Furthermore, the half-life of IL-8 mRNA for both ligands was similar ($<$30 minutes), suggesting that mRNA stability was not responsible for the differences in the kinetics of IL-8 accumulation between the 2 ligands. Transient transfection studies with reporter constructs containing 1.48 kb of the IL-8 promoter identified an Ox-PAPC–specific response region between -133 and -1481 bp of the IL-8 promoter. In contrast, TNF-α activation of the IL-8 promoter was mediated almost entirely through the nuclear factor-κB and activation protein-1 response elements present between -70 and -133 bp of the IL-8 promoter. Thus, although Ox-PAPC and TNF-α both induced IL-8 synthesis, our data suggest that the 2 ligands utilize different mechanisms in the regulation of IL-8 transcription. **(*Arterioscler Thromb Vasc Biol*. 2001;21:1585-1591.)**

We have previously shown that oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (Ox-PAPC), a major bioactive component of minimally modified LDL (MM-LDL), is present in atherosclerotic lesions and other sites of chronic inflammation.[1,2] Similar to other inflammatory cytokines such as tumor necrosis factor-α (TNF-α), Ox-PAPC activates endothelial cells to enhance monocyte-endothelial interactions partly through the induction of chemokines such as monocyte chemotactic protein-1 (MCP-1) and interleukin (IL)-8.[3,4] Interestingly, Ox-PAPC (or MM-LDL) but not TNF-α enhances monocyte-endothelial binding by mediating the activation of β1-integrins, resulting in deposition of the connecting segment-1 domain of fibronectin on the apical surface of endothelial cells.[5] On the other hand, TNF-α enhances endothelial-monocyte interactions through the induction of E-selectin and vascular cell adhesion molecule-1, which are not affected by Ox-PAPC.[6] Thus, Ox-PAPC and TNF-α utilize both similar and distinct induction patterns of gene expression to initiate endothelial-monocyte interactions. Although the molecular mechanisms of gene induction by TNF-α are well understood, the target receptors, signaling pathways, and molecular mechanisms by which Ox-PAPC (1) initiates functional changes in endothelial cells and (2) enhances endothelial-monocyte interactions are not known.

IL-8, a member of the CXC chemokine family, was initially characterized as a neutrophil chemotactic and activating factor.[7,8] IL-8 was subsequently identified to play an important role in many physiological and pathophysiological processes.[9,10] More recently, IL-8 has been implicated in

Received July 16, 2001; revision accepted July 27, 2001.

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This work was supported by National Institutes of Health (NIH) grant HL30568 and NIH training grant 5T32HL07895.

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monocyte activation and endothelial chemotaxis during angiogenesis.[11] IL-8 mRNA was found to be elevated in atherosclerotic lesions from atherectomized human carotid arteries as well as in macrophage foam cells in human atheroma.[12] Ox-LDL treatment and cholesterol loading induce IL-8 mRNA in macrophages.[13] Gerszten et al[14] demonstrated that IL-8 is critical for the firm adhesion of monocytes to activated endothelial cells. Bone marrow transplantation from mice lacking CXC receptor-2 (an orthologue for the human IL-8 receptor) into LDL receptor–deficient mice resulted in a reduction of atherosclerosis development in recipients' aortas.[15] These data suggest that IL-8 contributes to the development of atherosclerosis.

A number of ligands such as TNF-α and lipopolysaccharide induce IL-8 synthesis in a number of cell types, including endothelial cells.[16–18] IL-8 induction by TNF-α, IL-1β, IL-6, and lipopolysaccharide is regulated at the level of transcription.[18,19] The IL-8 promoter contains consensus binding sites for nuclear factor-κB (NF-κB), the CCAAT enhancer binding protein-β (C/EBPβ), and activation protein-1 (AP-1), all located in the proximal region between -70 and -133 bp of the human IL-8 promoter.[20] In macrophages and epithelial cells, TNF-α and other inflammatory cytokines activate the IL-8 promoter by cooperative binding of these 3 transcription factors to a composite enhanceosome within -70 to -133 bp of the proximal promoter.[21–24] Ox-PAPC and MM-LDL induce the accumulation of IL-8 protein in human aortic endothelial cell (HAEC) supernatants[3]; however, the mechanisms of Ox-PAPC–induced IL-8 synthesis are not known. In an effort to determine the molecular mechanisms by which Ox-PAPC induces IL-8 gene expression, we examined the regulation of IL-8 by Ox-PAPC and TNF-α in HAECs.

Methods

Reagents

Tissue culture media and reagents were obtained from Irvine Scientific Inc. Fetal bovine serum (FBS) was obtained from Hyclone. PAPC was purchased from Sigma, and Ox-PAPC was prepared as described previously.[2] Oxidation was monitored by mass spectrometry and terminated when >90% of PAPC was oxidized. Under these conditions, minimal formation of lysophosphatidylcholine was observed. Thus, the mass spectrometry profiles of the Ox-PAPC used in the present studies were similar to those previously shown.[2] Endotoxin concentrations in the phospholipid solutions were <0.1 pg/mL. TNF-α was purchased from R&D. AP-1 (5′-CTAGTGATGAGTCAGCCGGATC-3′), NF-κB (5′-GATCCAGGGGACTTTCCCTAGC-3′), and Oct-1 (5′-TGTCGAATGCAAATCACTAGA-3′) oligonucleotides for the electrophoretic mobility shift assay were purchased from Santa Cruz Biotechnology. Radioisotopes were purchased from Amersham Pharmacia Biotech. Dulbecco's modified Eagle's medium (DMEM)/high-glucose medium for HeLa cell culture and M199 medium for HAEC culture were purchased from Gibco/BRL.

Cell Culture

HAECs were isolated from the aortic rings of explanted donor hearts as described previously[1] and cultured in M199 medium supplemented with 20% (vol/vol) FBS, penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively; Gibco/BRL), sodium pyruvate (1 mmol/L), heparin (90 μg/mL, Sigma), and endothelial cell growth factor (20 μg/mL, Fisher Scientific). HeLa cells (American Type Culture Collection, Manassas, Va) were cultured in DMEM/high-glucose medium with 10% (vol/vol) FBS and penicillin-streptomycin.

Plasmids and Site-Directed Mutagenesis

Construction of luciferase reporter plasmids with a longer human IL-8 promoter (-1481 to $+44$ bp), a shorter IL-8 promoter (-133 to $+44$ bp), and multiple copies of individual NF-κB, C/EBPβ, or AP-1 elements has been described previously.[25] Mutations in the NF-κB and AP-1 elements in the context of the luciferase reporter construct containing the longer IL-8 promoter from -1481 to $+44$ bp (p[$-1481/+44$]-Luc) were created with a commercially available site-directed mutagenesis kit (QuickChange, Stratagene) and protocols provided by the manufacturer. Mutation primers of the 3 elements were designed as previously described[26,27] and customized from Invitrogen. The NF-κB element was mutated from TGAATTTCCT to TGGAATTT**aaa** and the AP-1 element, from TGACTCA to TGACT**gt**.

Transient Transfection

HeLa cells were plated in 6-well culture dishes (2×10^5 cells per well) in DMEM/high-glucose medium containing 10% FBS. All transfections were performed with a total of 1 μg per well of DNA with the Superfect Reagent (8 μL per well, Qiagen) according to the manufacturer's protocol. Four hours after transfection, cells were stimulated with either Ox-PAPC or TNF-α in DMEM/high-glucose medium/1% FBS. After 12 hours, total cell lysates were collected and analyzed for luciferase activity by using a luciferase assay system kit (Promega). Luciferase activity was normalized to that of cotransfected pSV–β-galactosidase (Promega).

Enzyme-Linked Immunosorbent Assay

IL-8 levels in HAEC supernatants were measured with an IL-8 ELISA kit (Quantikine, R&D) according to the manufacturer's protocol.

Northern Blotting

Total cellular RNA was isolated from HAECs and HeLa cells by using Trizol reagents (Gibco/BRL) according to the manufacturer's protocol. The human IL-8 cDNA probe (a 1.2-kb *Eco*RI fragment of human IL-8 cDNA) was radiolabeled with [α-^{32}P]dCTP by the random-priming method. A glyceraldehyde 3-phosphate dehydrogenase probe was included for normalization, and quantitative analyses were performed in all experiments with a phosphoimager (Kodak).

Extraction of Nuclear Proteins and Electrophoretic Mobility Shift Assay

HeLa cells were preincubated with 1% FBS–containing DMEM/high-glucose medium for 1 day. Cells were treated for 30 minutes with Ox-PAPC, TNF-α, and phorbol 12-myristate 13-acetate (Sigma). Cells were then harvested and resuspended in 50 μL of cell lysis buffer (10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L dithiothreitol [DTT], and 0.1% NP-40) and incubated on ice for 10 minutes. The cell lysates were mixed briefly and centrifuged at 10 000 rpm for 10 minutes at 4°C. Next, the pellets were resuspended in 15 μL of nuclei lysis buffer (20 mmol/L HEPES pH 7.9, 1.5 mmol/L $MgCl_2$, 5 mmol/L DTT, 0.5 mmol/L EDTA, 5 mmol/L PMSF, 25% glycerol, and 0.42 mol/L NaCl) and incubated on ice for 15 minutes. The nuclear lysates were mixed briefly and centrifuged at 14 000 rpm for 15 minutes at 4°C. The supernatants were collected and stored at -80°C for future electrophoretic mobility shift assay studies. Protein concentration was determined by the Bradford method.

The electrophoretic mobility shift assay was done as previously described.[28] In brief, oligonucleotides were end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase and purified in Microspin columns (Bio-Rad). For each 10-μL reaction, 8 μg of nuclear extract protein and 1 μL of purified labeled oligonucleotides were incubated with 1 μL of dI-dC (1 μg/μL) and 1 μL of 10× binding buffer (10 mmol/L Tris-HCl pH 7.5, 50 mmol/L NaCl, 0.5 mmol/L EDTA, 1 mmol/L $MgCl_2$, 0.5 mmol/L DTT, and 4% glycerol) at room temperature for 20 minutes. Samples were electrophoresed on a 4% nondenatured polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 100 V for 1 hour. The gels were dried and exposed to a ^{32}P phosphoimager for 2 hours to overnight, depending on the intensity of the radioactivity on the dried gels.

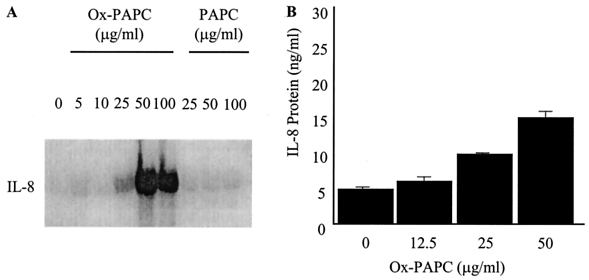

Figure 1. Ox-PAPC induces IL-8 message and protein. HAECs were treated with various concentrations of Ox-PAPC (0 to 100 μg/mL) and PAPC (0 to 100 μg/mL). Four hours later, total RNA and supernatant were collected to determine the levels of Ox-PAPC–induced IL-8 mRNA (A) and protein (B). Results are representative of 4 separate experiments; error bars were determined statistically with triplicates of the same condition in 1 experiment.

Results

Ox-PAPC Induced IL-8 mRNA and Protein Synthesis in HAECs

We have previously shown that Ox-PAPC induces the accumulation of IL-8 protein in HAEC supernatants.[3] To determine whether Ox-PAPC–induced accumulation of IL-8 protein was a result of new IL-8 mRNA synthesis, we performed Northern blotting analysis on HAECs treated with different concentrations of Ox-PAPC and PAPC. Ox-PAPC induced IL-8 mRNA synthesis in a dose-dependent fashion (Figure 1A). Untreated and PAPC-treated (up to 100 μg/mL) HAECs had very minimal, if any, IL-8 message (Figure 1A). Ox-PAPC also induced IL-8 protein accumulation in HAEC supernatants in a dose-dependent manner (Figure 1B).

Kinetics of IL-8 mRNA Accumulation Are Different in Ox-PAPC–Treated and TNF-α–Treated HAECs

We next examined the time course of TNF-α–induced and Ox-PAPC–induced IL-8 message accumulation in HAECs. Ox-PAPC–induced IL-8 transcripts were seen as early as 30 minutes (3-fold increase), peaked between 4 and 8 hours (≈60-fold increase), and decreased significantly by 24 hours (8-fold increase; Figures 2A and 2B). In contrast, TNF-α–induced IL-8 mRNA levels were maximal by 2 hours (300-fold induction) but returned to baseline by 6 hours (Figures 2A and 2B). In the presence of cycloheximide, IL-8 mRNA was still increased 65-fold, suggesting that new protein synthesis does not play a role in this prolonged induction by Ox-PAPC. IL-8 protein was detected in Ox-PAPC–treated HAEC supernatants as early as 2 hours, and the levels were still increasing at 24 hours (Figure 2C). TNF-α–induced IL-8 protein accumulated as early as 1 hour and peaked between 8 and 12 hours. There was no significant difference in the level of IL-8 after 12 hours (Figure 2D). These data suggest that IL-8 protein accumulation parallels IL-8 mRNA induction by the 2 agents.

Ox-PAPC Increased IL-8 mRNA Accumulation by Increasing Transcription

To determine whether IL-8 mRNA accumulation occurs at the level of transcription, HAECs were pretreated with actinomycin D (400 nmol/L) for 30 minutes and then treated with either Ox-PAPC (50 μg/mL) or TNF-α (20 ng/mL) for 2 hours. Northern blot analysis (Figure 3A) showed that actinomycin D completely inhibited IL-8 transcription by both Ox-PAPC and TNF-α (87% and 91% reduction, respectively; Figure 3B). To determine whether the differences in

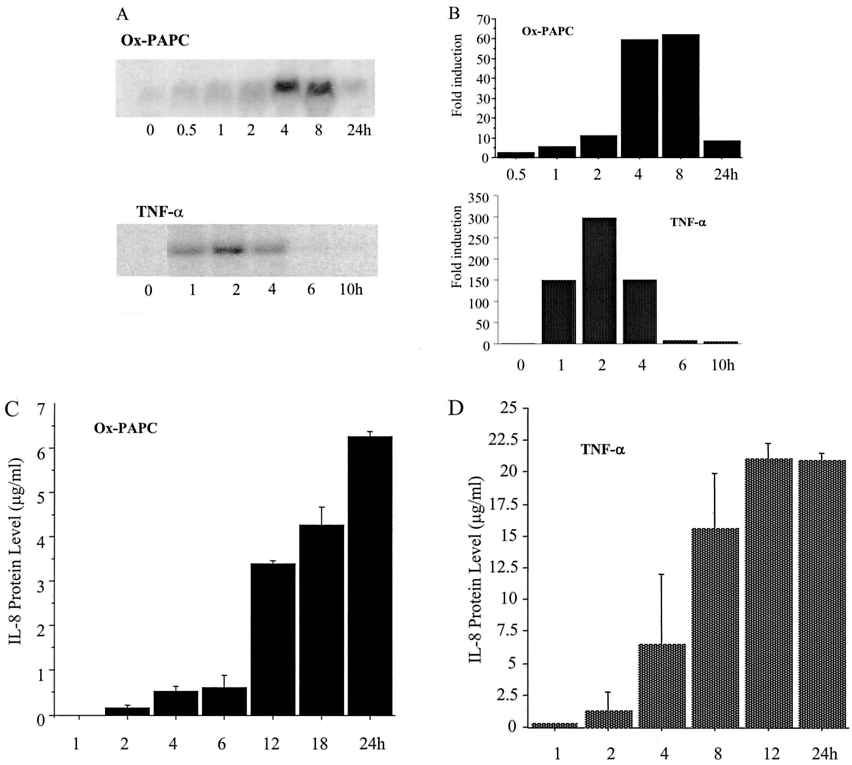

Figure 2. Ox-PAPC and TNF-α induce IL-8 mRNA and protein, but with different kinetics. HAECs were treated with either Ox-PAPC (50 μg/mL) or TNF-α (2 ng/mL). Total RNA and medium were collected at different time intervals (0 to 24 hours). A, Northern blots were performed with 5 μg of total RNA extracted from Ox-PAPC– (top panel) or TNF-α– (bottom panel) treated HAECs. B, Phosphoimage analysis of Northern blots of IL-8 mRNA induced by Ox-PAPC and TNF-α. All IL-8 message levels were normalized to glyceraldehyde 3-phosphate dehydrogenase levels. C and D, ELISAs were performed on HAEC supernatants to determine the levels of IL-8 protein synthesis induced by Ox-PAPC (C) and TNF-α (D) at different time interval (0 to 24 hours). Results are representative of 3 separate experiments.

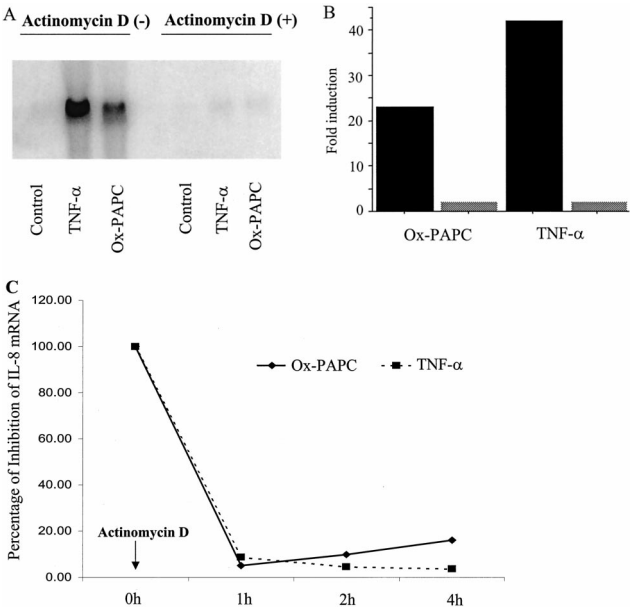

Figure 3. Ox-PAPC and TNF-α regulate IL-8 message at the level of transcription. HAECs were treated with or without actinomycin D (400 nmol/L) for 30 minutes and then with Ox-PAPC (40 μg/mL) or TNF-α (2 ng/mL). Two hours later, total RNA were extracted and IL-8 message induced by TNF-α and Ox-PAPC was determined by Northern blot (A) and quantified by phosphoimage analysis (B). All IL-8 message levels were normalized to glyceraldehyde 3-phosphate dehydrogenase levels. The stability of induced IL-8 mRNA by both ligands was determined by measuring the message half-life. HAECs were first treated with either Ox-PAPC or TNF-α. Two hours later, actinomycin D (400 nmol/L) was added to inhibit any further synthesis of new message. Total RNA was extracted at different time intervals (0, 1, 2, and 4 hours) after addition of actinomycin D. Northern blots and phosphoimager analysis were performed to quantify the levels of IL-8 mRNA (expressed as the percentage of IL-8 message amount at 0 hours) induced by Ox-PAPC (solid line) or TNF-α (dashed line). Results are representative of 2 separate experiments.

posttranscriptional regulation of IL-8 accounted for the differences in the kinetics of IL-8 induction by Ox-PAPC and TNF-α, HAECs were treated with either Ox-PAPC or TNF-α for 2 hours before actinomycin D (400 nmol/L) treatment, and IL-8 mRNA was quantified at various time points. The IL-8 mRNA transcript was completely degraded by 1 hour for both TNF-α and Ox-PAPC (Figure 3B), indicating that the half-life of IL-8 mRNA for both was similar (<30 minutes). These data suggest that both Ox-PAPC–induced and TNF-α–induced IL-8 expression is regulated at the level of transcription in HAECs (Figure 3C).

Ox-PAPC and TNF-α Activated Reporter Constructs Containing a 1.48-kb IL-8 Promoter

Both Ox-PAPC and TNF-α induced IL-8 protein synthesis in HeLa cells (data not shown); therefore, IL-8 promoter regulation study was performed in this easily transfected cell type. To determine whether Ox-PAPC can induce transcriptional activation of the human IL-8 promoter, HeLa cells were transiently transfected with a luciferase reporter plasmid containing −1481 to +44 bp of the human IL-8 promoter and were then treated with various concentrations of Ox-PAPC. Ox-PAPC induced luciferase activity in a dose-dependent fashion, suggesting that Ox-PAPC response elements are present in the first 1.48 kb of the IL-8 promoter (Figure 4).

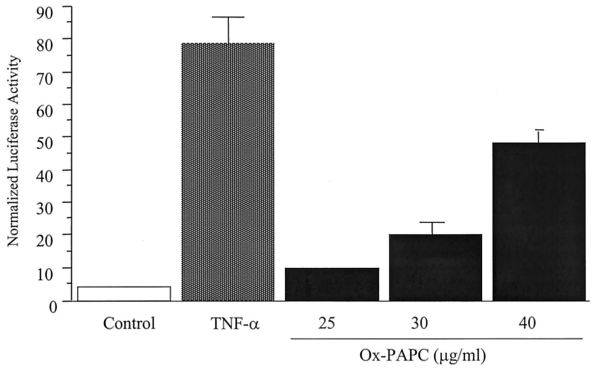

Figure 4. Ox-PAPC and TNF-α activate luciferase reporter constructs containing the IL-8 promoter. HeLa cells were transiently transfected for 2 hours with 0.8 μg per well of p(−1481/+44)-Luc, together with 0.2 μg per well of β-galactosidase expression plasmid, as described in Methods. Transfected cells were treated with various concentrations of Ox-PAPC (from 25 to 40 μg/mL) or TNF-α (2 ng/mL) for 12 hours. Cell lysates were collected and assayed for luciferase and β-galactosidase activities. Luciferase activities were expressed in arbitrary units after being normalized against β-galactosidase activities. Results are representative of 3 separate experiments.

Studies on NF-κB, AP-1, and Oct-1 Activation

To determine whether NF-κB, C/EBPβ, or AP-1 response elements participate in Ox-PAPC–mediated transcriptional activation, reporter constructs containing multiple NF-κB (p[NF-κB]$_3$-Luc), C/EBPβ (p[C/EBPβ]$_3$-Luc), or AP-1 (p[AP-1]$_3$-Luc) elements were transiently transfected into HeLa cells. TNF-α strongly activated p[NF-κB]$_3$-Luc by 7-fold, whereas Ox-PAPC had no effect (Figure 5A). On the other hand, Ox-PAPC mildly activated the p[AP-1]$_3$-Luc construct (Figure 5B), whereas TNF-α had no effect. Plasmid p[C/EBPβ]$_3$-Luc was not activated by either TNF-α or Ox-PAPC (data not shown). Furthermore, nuclear extracts from cells treated with TNF-α but not Ox-PAPC showed increased binding activity to a [γ-^{32}P]ATP-labeled NF-κB consensus sequence from the human IL-8 promoter (Figure 5C). TNF-α did not affect AP-1 binding activity in HeLa nuclear extracts, whereas a minimal increase was seen with Ox-PAPC (Figure 5C). Phorbol 12-myristate 13-acetate, an AP-1 activator, served as a positive control. Previous studies had shown that the induction of IL-8 may be the result of downregulation of Oct-1, a constitutive repressor of the C/EBPβ response element.[29] Because Ox-PAPC did not induce NF-κB and only minimally induced AP-1 activity, we hypothesized that Oct-1 binding activity might be decreased by Ox-PAPC and result in induction of IL-8. However, nuclear extracts from HeLa cells treated with Ox-PAPC or TNF-α showed no decrease in Oct-1 binding activity on the electrophoretic mobility shift assay (Figure 5C).

An Ox-PAPC–Responsive Element Is Located in the Upstream Region of the Human IL-8 Promoter

To determine the sequences in the IL-8 promoter important for Ox-PAPC–induced transcription, reporter constructs containing different lengths of the IL-8 promoter (−1481 to +44 and −133 to +44) were transiently transfected into HeLa cells. The IL-8 promoter construct containing p[−1481/+44]-Luc was activated by Ox-PAPC; however, the construct containing p[-133/+44]-Luc was only mildly activated

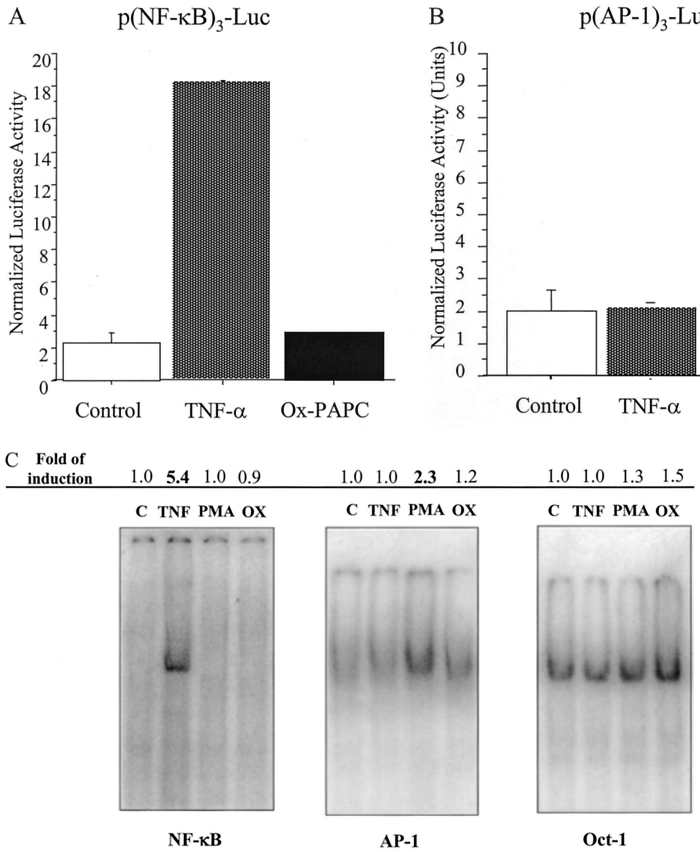

Figure 5. Activation of NF-κB, AP-1, and Oct-1. HeLa cells were transiently transfected with 0.8 μg of (A) p[NF-κB]$_3$-Luc or (B) p[AP-1]$_3$-Luc as per the aforementioned protocol in the legend to Figure 4. Transfected cells were treated with either 30 μg/mL Ox-PAPC (solid black bar) or 2 ng/mL TNF-α (dotted bar) for 12 hours. Results were shown in luciferase activity units that were normalized against β-galactosidase activities. p[NF-κB]$_3$-Luc is a luciferase reporter construct with 3 copies of the NF-κB responsive element and p[AP-1]$_3$-Luc, with 3 copies of the AP-1 responsive element. C, HeLa cell nuclear extracts were harvested after 30 minutes of stimulation with Ox-PAPC (40 μg/mL), TNF-α (2 ng/mL), phorbol 12-myristate 13-acetate (PMA, 20 ng/mL), or control media (C). [γ-^{32}P]ATP-labeled NF-κB, AP-1, and Oct-1 oligonucleotides were individually incubated with 5 μg of nuclear extracts at room temperature for 20 minutes. Reaction products were run on nondenatured gels and exposed to phosphoimage analysis. These image values were used to calculate the fold induction (above lanes) compared with untreated cells, and all values obtained were well within the linear range of phosphoimage measurement. Results are representative of 2 separate experiments.

by Ox-PAPC (Figure 6B). In contrast, TNF-α activated both the longer p[−1481/+44]-Luc and the shorter p[−133/+44]-Luc constructs (>17-fold, Figure 6A). Thus, deletion of the upstream region of the IL-8 promoter from −1481 to −133 bp resulted in a 75% reduction in promoter activation by Ox-PAPC (Figures 6A and 6B), suggesting that an Ox-PAPC–response region resides between −1481 and −133 bp of the human IL-8 promoter. Individual mutations in the conserved response elements (NF-κB and AP-1) generated in the context of the longer construct p[−1481/+44]-Luc] resulted in significant inhibition of TNF-α–induced IL-8 promoter activation but only slightly decreased Ox-PAPC–induced activation (Figures 6A and 6B).

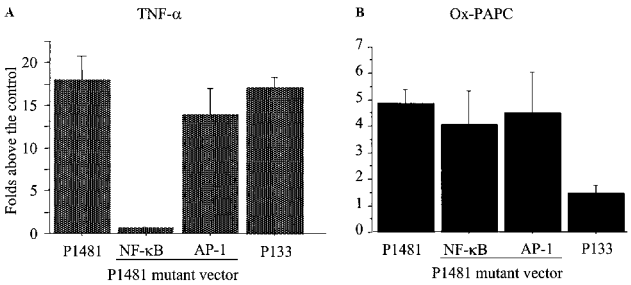

Figure 6. Ox-PAPC mediates IL-8 promoter activation through an Ox-PAPC–specific response region between −133 and −1481 bp of the IL-8 promoter. HeLa cells were transiently transfected with 2 luciferase reporter plasmids, p[−1481/+44]-Luc and p[−133/+44]-Luc, or with 3 separate p[−1481/+44]-Luc constructs containing site-directed mutations of the NF-κB or AP-1 element, as described in Methods. Transfected cells were treated with either 30 μg/mL Ox-PAPC (A) or 1 ng/mL TNF-α (B). Results are shown as the fold induction above control. P-1481 indicates p[−1481/+44]-Luc; P-133, p[−133/+44]-Luc. Results are representative of 3 separate experiments.

Discussion

This study is the first to examine the mechanism by which Ox-PAPC, an oxidatively fragmented lipid component of MM-LDL, regulates transcription. In this study, Ox-PAPC was compared with the more thoroughly characterized regulation of IL-8 by TNF-α. Our results demonstrate that the major effect of Ox-PAPC on the IL-8 promoter is involved with an upstream sequence between −1481 and −133 bp. There are several responsive elements present in this region of the human IL-8 promoter that have been known for years, eg, the glucocorticoid responsive element and the hepatocyte nutrition factor-1 binding site, which are located at positions −330 to −325 bp and −381 to −376 bp, respectively, upstream from the TATA box.[20] Neither has been demonstrated to have a transcription-enhancing property; in fact, activated glucocorticoid responsive element mediates the downregulation of NF-κB–activated transcription by interfering with the binding of NF-κB p65 to its *cis*-element[30] or by directly interfering with transactivation of the NF-κB p65 subunit.[31] There is no other *cis*-element in the human IL-8 promoter that has been described. Thus, we propose that there is a novel *cis*-element located in the 5′-flanking region between −1481 and −133 bp of the human IL-8 promoter. One likely candidate for this putative distant enhancer may be the peroxisome proliferator–activated receptor response element (PPRE).

Recently, work from our laboratory has shown that MM-LDL and Ox-PAPC activate PPRE and peroxisome proliferator–activated receptor-α activators and stimulate the production of MCP-1 and IL-8 in HAECs.[3] Additionally, the PPRE has been reported to function as an indispensable *cis*-enhancing element that may be located as far as −2 kb

upstream from the transcription initiation site.[32] These observations support the notion that the PPRE may be the *cis*-enhancing element responsive to Ox-PAPC in the 5′-flanking sequence of the human IL-8 promoter.

In our current study, by using both gel shift assays and transient transfection experiments, neither the NF-κB nor AP-1 signaling pathway was activated by Ox-PAPC. This result is different from previous data showing activation of NF-κB and AP-1 signaling by Ox-LDL,[33–35] MM-LDL,[36] or oxidized phospholipids, such as platelet-activating factor–like lipids.[37] Activation of NF-κB by Ox-LDL was demonstrated to be a result of enhanced phosphorylation of inhibitor-κB in monocytes.[34] Platelet-activating factor–like lipids, enzymatically oxidative fragmentation products of ether-containing phosphatidylcholine, activate NF-κB signaling in the cells expressing platelet-activating factor receptors, such as monocytes.[37] MM-LDL was also shown by electrophoretic mobility shift assay to activate binding to an NF-κB consensus sequence.[36] Although Ox-PAPC activates the AP-1 element in isolation, it only minimally increased AP-1 binding on the electrophoretic mobility shift assay and activated the longer IL-8 promoter with the AP-1 mutation. Thus, we have demonstrated that unlike Ox-LDL, the AP-1 element is not important in the human IL-8 promoter activation by Ox-PAPC.

The difference in the effects of Ox-LDL, MM-LDL, platelet-activating factor–like lipids, and Ox-PAPC may be related to the different cell types used for these studies. Furthermore, they may be related to the different bioactive lipid components in Ox-PAPC versus Ox-LDL and platelet-activating factor–like lipids. We found that 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine, an oxidative fragmented product purified from Ox-PAPC, is the major bioactive lipid that enhances monocyte binding to endothelial cells.[38] The level of this phospholipid is quite low in Ox-LDL; furthermore, Ox-PAPC, being ester derived, does not contain platelet-activating factor–like lipids. In a separate study, it will be shown that 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine and its dehydration product are the lipids in Ox-PAPC that are mainly responsible for inducing IL-8 in HAECs.

In summary, this current study has provided information on the mechanism by which Ox-PAPC induces IL-8 transcription. We have demonstrated that Ox-PAPC transcriptionally regulates IL-8 and causes a prolonged increase in transcription, compared with TNF-α. This prolonged increase was not secondary to new protein synthesis. We have also shown that the induction of IL-8 by Ox-PAPC, unlike that by Ox-LDL or TNF-α, is not mediated by way of the NF-κB signaling pathway. IL-8 upregulation by Ox-PAPC is also not a result of activation of NF-κB, C/EBPβ, and AP-1 or of downregulation of a constitutive transcriptional repressor Oct-1. Rather, we have demonstrated that Ox-PAPC induces IL-8 expression by activation of a putative *cis*-enhancer element in the upstream IL-8 promoter sequence. Our results showing differential regulation of the IL-8 promoter by Ox-PAPC compared with that by TNF-α provide a basis for the differences previously reported in genes activated by the 2 agents.

Acknowledgment

We thank Dr Yin Tintut for her technical support in several key experiments in this work.

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