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Analysis of inflammatory gene induction by oxidized phospholipids in vivo by quantitative real-time RT-PCR in comparison with effects of LPS

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Abstract

Oxidized phospholipids are thought to play a role in the development of atherosclerosis and other chronic inflammatory processes. In this study, we analyzed the expression of inflammatory genes induced by oxidized L-α-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholin (OxPAPC) in vitro and in vivo using quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Cultured human umbilical vein endothelial cells (HUVEC) and monocyte-like U937 cells were treated with OxPAPC or lipopolysaccharide (LPS) for 3 h. For in vivo studies, OxPAPC or LPS was injected intravenously into female C57Bl/6J mice and different tissues were isolated after 3 h. We found that both OxPAPC and LPS induced expression of early growth response factor 1 (EGR-1) and monocyte chemoattractant protein 1 (MCP-1) in HUVEC and of JE, the mouse homologue of MCP-1, in liver and heart. Interestingly, OxPAPC but not LPS increased expression of heme oxygenase 1 (HO-1) in U937 cells, HUVEC, aorta, heart, liver, and isolated blood cells. In contrast, E-selectin was selectively induced by LPS, but not by OxPAPC. Finally, OxPAPC-induced expression of HO-1 was blocked by a platelet-activating factor (PAF) receptor antagonist. We conclude that oxidized phospholipids are biologically active in vivo and exert a specific response inducing a pattern of genes that is different from that induced by LPS. In addition, we demonstrate that the quantitative real-time RT-PCR technology is a proper tool to investigate differential inflammatory gene induction in vivo.

Keywords: Oxidized phospholipids; Inflammation; Atherosclerosis; Real-time RT-PCR

1. Introduction

There is increasing evidence that lipid oxidation products may play an important role in the pathogenesis of atherosclerosis as well as other chronic inflammatory diseases (Lusis, 2000). Oxidized lipids were shown to activate cells of the vascular wall as well as blood cells to induce expression of inflammatory genes. However, there are striking differences from cell activation by other proinflammatory mediators such as interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), or lipopolysaccharide (LPS). These mediators activate the classical nuclear factor kappa B (NFκB) pathway leading to an acute inflammatory response by elevating the expression of adhesion molecules like E-selectin, VCAM-1, or intercellular adhesion molecule 1 (ICAM-1), resulting in adhesion of monocytes as well as neutrophils to the site of inflammation. Oxidized lipids, however, stimulate endothelial cells to specifically bind monocytes, but not neutrophils, a hallmark of atherosclerosis and other chronic inflammatory diseases (Lusis, 2000).

We were interested in the mechanisms of endothelial activation leading to specific monocyte adhesion after stimulation with various oxidized lipids. We could show

Abbreviations: HUVEC, human umbilical vein endothelial cells; PAPC, L-α-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; EGR-1, early growth response factor 1; TF, tissue factor; HO-1, heme oxygenase 1; MM-LDL, minimally modified low-density lipoprotein; PKC, protein kinase C; PAF-AH, platelet-activating factor acetylhydrolase; Group II $sPLA_2$, group II secretory phospholipase A_2; RT-PCR, reverse transcriptase-polymerase chain reaction; IL, interleukin; PON, paraoxonase; NFκB, nuclear factor κ B; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; MEK, MAPK/ERK kinase; NFAT, nuclear factor of activated T cells; TNF-α, tumor necrosis factor α; ICAM, intercellular adhesion molecule; u-PA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1.

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that different lipid oxidation products, such as oxidized phospholipids (Berliner et al., 1990; Watson et al., 1995), oxidized cholesteryl esters (Huber et al., 2002), and isoprostanes (Leitinger et al., 2001), were capable of inducing specific monocyte adhesion to endothelial cells. Activation of the mitogen-activated protein kinase (MAPK) signaling cascade, rather than the classical NFκB pathway, was shown to be important for this effect (Huber et al., 2002; Leitinger et al., 2001). Furthermore, tissue factor (TF) expression induced by oxidized L-α-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholin (OxPAPC) was mediated by the protein kinase C (PKC)/MAPK and ERK kinase (MEK)/extracellular signal-regulated protein kinase (ERK)/early growth response factor 1 (EGR-1) and Ca^{2+}/calcineurin/nuclear factor of activated T cell (NFAT) pathways, not involving the NFκB pathway (Bochkov et al., 2002). In addition, OxPAPC-induced expression of IL-8 was mediated by a pathway independent of NFκB, C/EBPβ, and AP-1 (Yeh et al., 2001). Moreover, there is evidence that LPS-induced NFκB-mediated inflammation is down-regulated by OxPAPC (Leitinger et al., 1999a), leading to the hypothesis that lipid oxidation products may promote the shift from an acute inflammatory response to a chronic state. However, in order to prove such a hypothesis, we need to know more about the specific inflammatory response that is elicited by oxidized lipids, especially in vivo.

It is believed that lipid oxidation products exert their biological activity towards vascular cells only locally, e.g., after they accumulate in the subendothelial space during atherogenesis. However, we have shown that in addition to oxidized low-density lipoprotein (LDL), there exist other sources for lipid oxidation products: activated cells as well as cells undergoing apoptosis shed parts of their membranes in the form of vesicles. These membrane vesicles also contain oxidized lipids that are capable of inducing monocyte–endothelial cell interactions (Huber et al., 2002). However, whether these lipids can exert their biological activity in vivo in the bloodstream remains speculative for the following reason: plasma enzymes such as paraoxonase (PON), platelet-activating factor acetylhydrolase (PAF-AH), or secretory nonpancreatic phospholipase A_2 (group II $sPLA_2$) have been shown to destroy biologically active oxidized phospholipids (Leitinger et al., 1999b; Shih et al., 1998; Watson et al., 1995). Increased activity of these enzymes inhibited the development of atherosclerosis (Shih et al., 1998).

Oxidized phospholipids were shown to be the biologically active components in minimally modified LDL (MM-LDL) (Watson et al., 1995) and to induce expression of inflammatory genes in vitro in various cell types. A specific receptor for the action of oxidized phospholipids has not been identified yet. However, it has been shown that the effects of oxidized phospholipids can be blocked by PAF receptor antagonists in vitro and in vivo (Leitinger et al., 1997; Subbanagounder et al., 1999).

Among inflammatory genes induced by oxidized lipids, monocyte chemoattractant protein 1 (MCP-1) was shown to be induced in human aortic endothelial cells and smooth muscle cells by MM-LDL (Cushing et al., 1990) and OxPAPC (Reddy et al., 2001; Wang et al., 1994). The mouse homologue of MCP-1 has been identified as JE (Rollins et al., 1989). In vivo experiments showed that JE is induced in the liver of mice, either by intravenous injection of MM-LDL (Liao et al., 1991) or by feeding a high-fat, high-cholesterol diet (Liao et al., 1993).

Recently, we could show that oxidized phospholipids increase synthesis of EGR-1 in vitro in human umbilical vein endothelial cells (HUVEC; Bochkov et al., 2002). *Egr-1* is known to be up-regulated by growth factors, cytokines, hypoxia, physical forces, and injurious stimuli, and high levels of EGR-1 were found in atherosclerotic lesions (Khachigian et al., 1996; McCaffrey et al., 2000). Oxidized lipids thus may contribute to increased levels of EGR-1 in atherosclerotic lesions. One of the genes that are regulated by EGR-1 is TF. Moreover, TF was also shown to be up-regulated by oxidized lipids (Bochkov et al., 2002; Drake et al., 1991; Penn et al., 1999, 2000). In contrast to the up-regulation of proinflammatory genes, oxidized phospholipids were also shown to induce the expression of protective enzymes such as heme oxygenase 1 (HO-1) (Ishikawa et al., 1997), which is the rate-limiting enzyme in heme catabolism and has antioxidative capacity (Maines, 1988).

The aim of this study was to examine whether oxidized phospholipids are biologically active in vivo in mice after intravenous injection. Thus, we tested the effects of OxPAPC and LPS on the expression of MCP-1/JE, EGR-1, TF, and HO-1 in vivo. We show that effects induced by oxidized phospholipids are specific for different organs, can be blocked by a PAF receptor antagonists, and differ from LPS-induced effects. Finally, we demonstrate that the method of quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) is a proper tool to detect differential regulation of gene expression in response to different stimuli.

2. Materials and methods

2.1. Materials

Trizol and primers were obtained from Life Technologies. Gene Amp RNA PCR kit core was purchased from Perkin Elmer. PAPC, LPS (from *Escherichia coli* serotype 055:B5), NaCl solution, and tissue culture media were from Sigma (St. Louis, MO). RNAlater, RNAqueous 4PCR, RNAqueous kit, and RNAqueous blood module were purchased from Ambion (Austin, TX, USA). CV-3988, a PAF receptor antagonist, was obtained from Biomol Research Laboratories (Plymouth, PA). Capillaries and Fast Start DNA Master SYBR Green I kit were from Roche Diagnostics.

2.2. Lipid oxidation

PAPC was oxidized by exposure of dry lipid to air for 72 h. The extent of oxidation was monitored by positive ion electrospray mass spectrometry as described previously (Watson et al., 1997). OxPAPC used for experiments contained less than 50 pg/ml of endotoxin as determined by the *Limulus* amebocyte assay (Bio Whittaker, Walkervill, MD).

2.3. Tissue culture

HUVEC and monocyte-like U937 cells were cultured at 37 °C and 5% CO_2 either in medium 199 (for HUVEC) or in RPMI 1640 (for U937 cells) containing 20% supplemented calf serum (SCS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. HUVEC at passage 3 were grown to confluence in six-well plates, and experiments with U937 cells were performed in six-well plates (5×10^5 cells/well). Cells were stimulated for 3 h in culture media containing 5% serum with 50, 75, 100, or 150 μg/ml OxPAPC or 10 μg/ml LPS in quadruplicates.

2.4. Animals

Mice were purchased from the Forschungsinstitut für Versuchstierzucht und-haltung (Himberg, Austria). Mice were kept under pathogen-free conditions. OxPAPC (100, 250, and 500 ng), LPS (50 μg), and/or CV-3988 (3 mg/kg; 60 ng/mouse) were injected in 200 μl NaCl solution (0.9%) into the tail vein of 6–9-week-old female C57Bl/6J mice (~20 g). For negative control, mice received 200 μl of saline solution. After 3 h, animals were sacrificed, the whole blood, liver, aorta, lung, and heart were collected, later mixed gently in RNA, and stored at 4 °C overnight prior to isolation of RNA. Each treated group consisted of eight animals.

2.5. RNA isolation and cDNA preparation

RNA was isolated either using Trizol reagent (for liver, heart, lung, and in vitro experiments), with RNAqueous for PCR kit (for aorta), or with RNAqueous blood module kit (for whole blood) according to the protocols of the manufacturers. Nine hundred nanograms of total RNA were reverse transcribed with MuLV-RT using Oligo dT (16) primers.

2.6. Quantitative RT-PCR

2.6.1. Design of primers

The cDNA sequences of the investigated genes were obtained from GenBank (for accession numbers, see Table 1). The primer for β2 microglobulin (β2M) was described by Wellmann et al. (2001). All other PCR primers (see Table 1) were designed using the PRIMER3 software from the Whitehead Institute for Biomedical Research (Mayrand et al., 1991) (code is available at, http://www-genome.wi.mit.edu/genome_software/other/primer3.html).

Table 1

Primer, accession number of target gene	Sequence (5′–3′)	RT-PCR efficiency
HS β2M (Wellmann et al., 2001)	f: GATGAGTATGCCTGCCGTGTG r: CAATCCAAATGCGGCATCT	2.198
HS MCP-1 (Chen et al., 2001)	f: AAGATTGCCCAGAAAGCCCTGGAC r: AACTGTCGCCACCAGAAAGCTGAG	2.232
HS HO-1 (Chen et al., 2001)	f: AAGATTGCCCAGAAAGCCCTGGAC r: AACTGTCGCCACCAGAAAGCTGAG	1.868
HS EGR-1, NM 001964	f: CAGCACCTTCAACCCTCAG r: CACAAGGTGTTGCCACTGTT	1.812
HS E-selectin, NM 000450	f: GGTTTGGTGAGGTGTGCTC r: TGATCTTTCCCGGAACTGC	1.849
HS PAI-1, J03764	f: CAGACCAAGAGCCTCTCCAC r: ATCACTTGGCCCATGAAAAG	2.519
HS TF, NM 010171	f: CCGAACAGTTAACCGGAAGA r: TCAGTGGGGAGTTCTCCTTC	1.749
MM β2M, NM 009735	f: ATTCACCCCCACTGAGACTG r: TGCTATTTCTTTCTGCGTGC	1.923
MM JE (Barber et al., 1999)	f: CTTCTGGGCCTGCTGTTCA r: CAAGCCTACTCATTGGGATCA	2.01
MM HO-1, NM 010442	f: GCCACCAAGGAGGTACACAT r: GCTTGTTGCGCTCTATCTCC	1.794
MM EGR-1, NM 007913	f: CAGGAGTTGGAGTGTTGTGG r: TATCCCATGGGCAATAGAGC	1.931
MM E-selectin, NM 011345	f: AGCTACCCATGGAACACGAC r: CGCAAGTTCTCCAGCTGTT	2.193
MM PAI-1, M33960	f: GTCTTTCCGACCAAGAGCAG r: ATCACTTGCCCCATGAAGAG	2.066
MM TF, NM 010171	f: ATGTGACCTGGGCCTATGAA r: TTACTGGCTGTCCGAGGTTT	1.886

The amplified cDNA regions were chosen to span one or more large introns in the genomic sequence, thus avoiding coamplification of genomic DNA under our amplification protocol. The testing of primer specificity included melting point analyses, agarose gel electrophoresis of the PCR products, and subsequent DNA sequencing.

2.6.2. Real-time RT-PCR

Quantitative RT-PCR was performed by LightCycler technology (Roche Molecular Biochemicals) using SYBR Green I detection. In all assays, cDNA was amplified using a standardized program (10′ denaturing step; 55 cycles of 5″ at 95 °C, 15″ at 65 °C, and 15″ at 72 °C; melting point analysis in 0.1 °C steps; final cooling step). Each LightCycler capillary was loaded with 1.5 μl DNA Master Mix, 1.8 μl $MgCl_2$ (25 mM), 10.1 μl H_2O, and 0.4 μl of each primer (10 μM). The final amount of cDNA per reaction corresponded to 2.5 ng of total RNA used for reverse transcription. Relative quantification of target gene expression was performed using a mathematical model (Pfaffl, 2001), which is also recommended by Roche Molecular Biochemicals. The relative expression ratio is calculated from the real-time PCR efficiencies and the crossing point deviation of an unknown sample versus control. The PCR efficiencies for reference and target molecules (amplicons) were calculated using the Relative Quantification Software from Roche Molecular Biochemicals (Version 1.0). Dilution series of samples over four orders of magnitude (from 25 to 2.5 pg/reaction) were used for each primer pair to determine real-time PCR efficiency for one cycle in the exponential phase. Efficiencies for one cycle in the exponential phase were calculated from the slopes indicated by the software using the formula $E=10^{[-1/\text{slope}]}$ (Table 1). The expression of the reference molecule was corrected for β2M (Koenig et al., 2001; Wellmann et al., 2001). The equation for relative expression of a target gene in an "induced" sample versus an "uninduced" control is:

$$E_T^{CpT(C)-CpT(S)} \times E_R^{CpR(S)-CpR(C)}$$

where E_T is the PCR efficiency of the target molecule (e.g., MCP-1, EGR-1), E_R is the PCR efficiency of the reference molecule (β2M), CpT(C) is the crossing point value of the target molecule in the (uninduced) control, CpT(S) is the crossing point value of the target molecule in the (induced) sample, CpR(S) is the crossing point value of the reference molecule in the (induced) sample, and CpR(C) is the crossing point value of the reference molecule in the (uninduced) control.

For CpT(C) and CpR(C), the mean values of the control (uninduced) group were used (in vitro, n=4; in vivo, n=8). Results are shown as mean values±standard error.

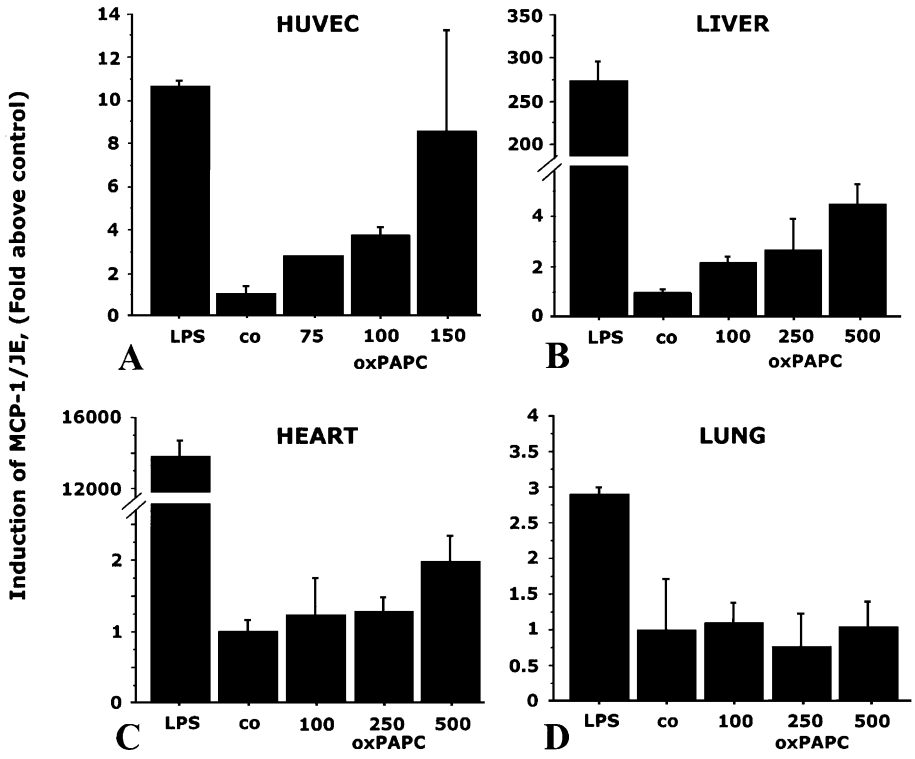

Fig. 1. MCP-1/JE is expressed in vitro and in vivo after stimulation with OxPAPC. Confluent HUVEC were incubated with 10 μg/ml LPS, medium only (co), or indicated concentrations (μg/ml) of OxPAPC. RNA was isolated 3 h after stimulation, reverse transcribed, and quantitative real-time RT-PCR was performed for quantitation of MCP-1 expression as described in Materials and Methods (A). Mice were injected with 50 μg of LPS, saline (co), or indicated concentrations of OxPAPC (μg). Organs were taken after 3 h, RNA was isolated, reverse transcribed, and quantitative real-time RT-PCR was performed to quantitate JE expression as described in Materials and Methods (B: liver, C: heart, D: lung).

3. Results

3.1. MCP-1/JE is up-regulated in HUVEC and in liver after treatment with OxPAPC

It was shown previously that MM-LDL induced the expression of JE in the liver (Liao et al., 1991) and of MCP-1 in endothelial and smooth muscle cells (Cushing et al., 1990). OxPAPC, a component of MM-LDL, was shown to be responsible for the biological activity of MM-LDL (Watson et al., 1995). To establish the method of quantitative real-time RT-PCR to investigate OxPAPC-induced gene regulation, expression of MCP-1 in HUVEC and in different organs from mice treated with OxPAPC or LPS was examined after 3 h of treatment. First, JE expression in liver and MCP-1 expression in HUVEC were analyzed.

Besides the strong gene up-regulation by LPS, we observed a dose-dependent increase in MCP-1 in HUVEC (Fig. 1A) and JE expression in liver (Fig. 1B) induced by OxPAPC. Furthermore, JE expression was slightly increased in the heart after OxPAPC treatment; however, strong induction was observed after LPS treatment (Fig. 1C). Lung (Fig. 1D), aorta, and isolated blood cells did not show increased expression of JE in response to OxPAPC (data not shown), while there was a strong response induced by LPS in lung (Fig. 1D) and blood cells (data not shown). Interestingly, aortas of LPS-treated mice showed only a weak induction of JE (data not shown). In addition, MCP-1

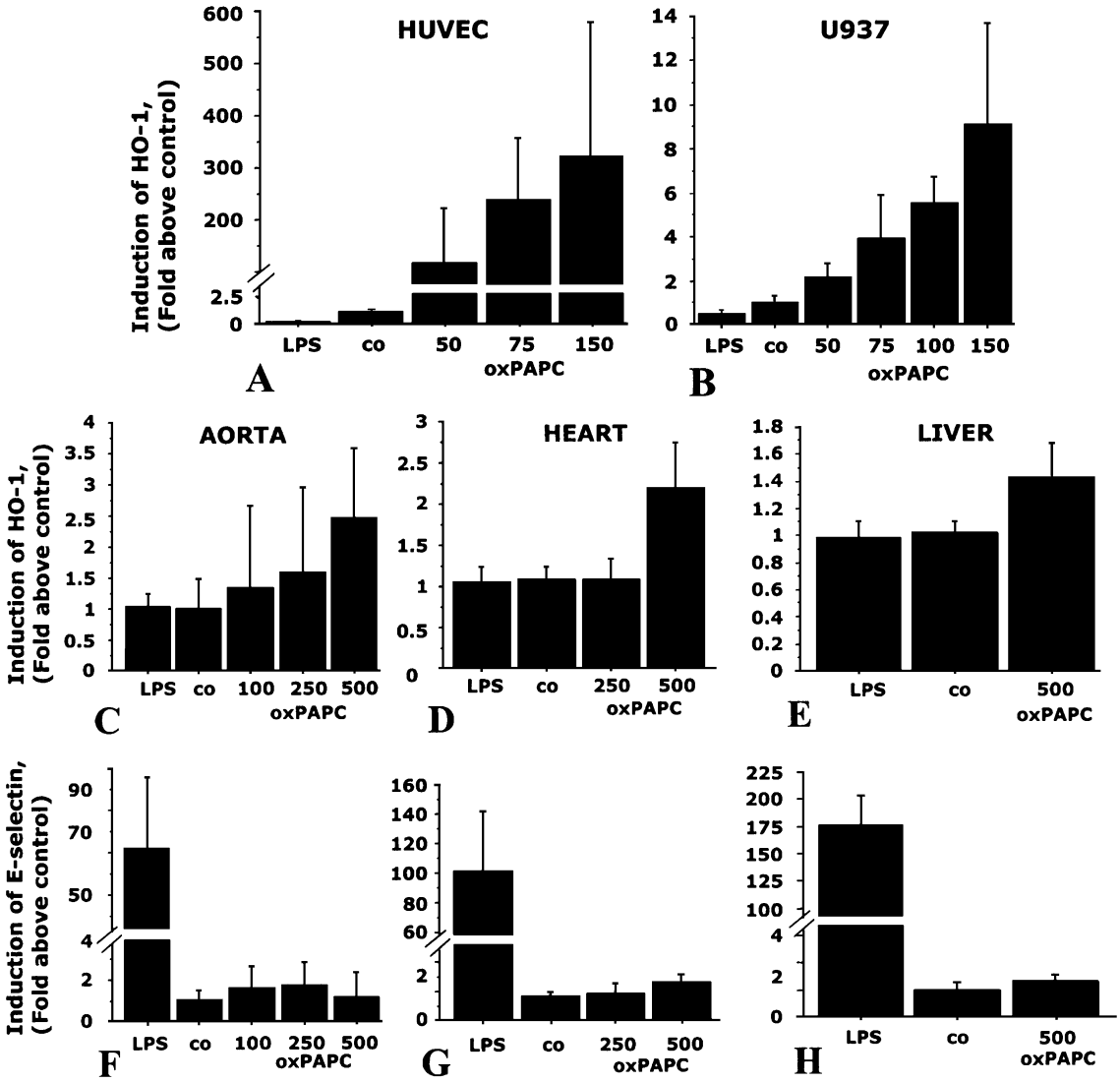

Fig. 2. Expression of HO-1 is induced by OxPAPC and E-selectin is induced by LPS. Confluent HUVEC or U937 cells in suspension were incubated with 10 μg/ml LPS, medium only (co), or indicated concentrations (μg/ml) of OxPAPC. RNA was isolated 3 h after stimulation, reverse transcription, and quantitative real-time RT-PCR was performed for quantitation of HO-1 expression as described in Materials and Methods (A: HUVEC, B: U937). Mice were injected with 50 μg of LPS, saline (co), or indicated concentrations of OxPAPC (μg). Organs were taken after 3 h, RNA was isolated, reverse transcribed, and quantitative real-time RT-PCR was performed to quantitate HO-1 and E-selectin expression as described in Materials and Methods (HO-1: C: aorta, D: heart, E: liver; E-selectin: F: aorta, G: heart, H: liver).

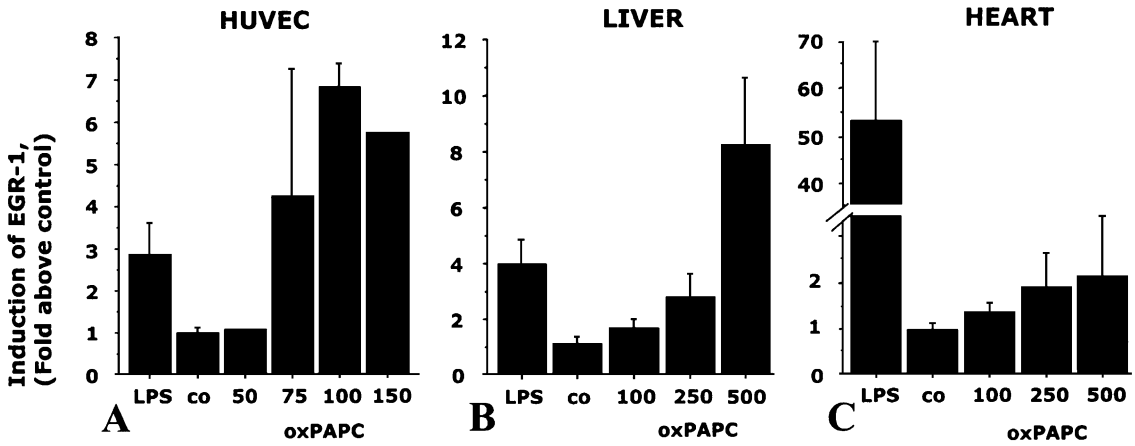

Fig. 3. EGR-1 is up-regulated in HUVEC, liver, and heart by OxPAPC. Confluent HUVEC were incubated with 10 μg/ml LPS, medium only (co), or indicated concentrations (μg/ml) of OxPAPC. RNA was isolated 3 h after stimulation, reverse transcription, and quantitative real-time RT-PCR was performed for quantitation of EGR-1 expression as described in Materials and Methods (A). Mice were injected with 50 μg of LPS, saline (co), or indicated concentrations of OxPAPC (μg). Organs were taken after 3 h, RNA was isolated, reverse transcribed, and quantitative real-time RT-PCR was performed to quantitate EGR-1 expression as described in Materials and Methods (B: liver, C: heart).

was up-regulated in U937 cells by LPS but not by OxPAPC (data not shown).

3.2. Effects of OxPAPC are specific and different from those induced by LPS

To determine whether effects induced by OxPAPC and LPS were specific for different genes, we compared the induction of E-selectin and HO-1 expression in different cell types in vitro and in various organs in vivo after treatment with OxPAPC and LPS. OxPAPC induced HO-1 expression in a dose-dependent manner in HUVEC and U937 cells, while treatment with LPS showed no response in both cell types (Fig. 2A and B). In contrast, OxPAPC did not induce E-selectin expression in HUVEC, while LPS strongly induced E-selectin expression (data not shown). In vivo, OxPAPC induced increased expression of HO-1 in aorta, heart, liver (Fig. 2C–E), and blood cells (Fig. 5), while LPS was inactive. In contrast, E-selectin expression in these tissues was only induced by LPS, but never by OxPAPC (Fig. 2F–H). In addition, plasminogen activator inhibitor type 1 (PAI-1) expression in liver was induced by LPS, but not by OxPAPC (not shown). These findings demonstrate that both HO-1 and E-selectin gene expression are differentially regulated by OxPAPC and LPS, both in vitro and in vivo.

3.3. Expression of egr-1 is up-regulated by OxPAPC in vivo

EGR-1 is thought to be an important transcription factor involved in gene induction during chronic inflammation such as atherosclerosis (Silverman and Collins, 1999). We have shown previously that EGR-1 expression is increased in HUVEC treated with oxidized phospholipids (Bochkov et al., 2002). Here we show that OxPAPC induced *egr-1* gene expression in HUVEC in a concentration-dependent manner (Fig. 3A), whereas in U937 cells *egr-1* expression

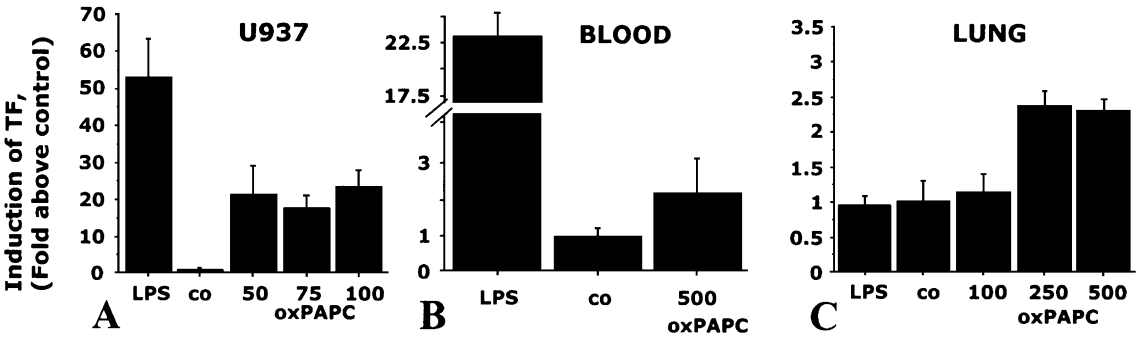

Fig. 4. TF expression is induced by OxPAPC in vitro and in vivo. Monocyte-like U937 cells were incubated with 10 μg/ml LPS, medium only (co), or indicated concentrations (μg/ml) of OxPAPC. RNA was isolated 3 h after stimulation, reverse transcription, and quantitative real-time RT-PCR was performed for quantitation of TF expression as described in Materials and Methods (A). Organs were taken after 3 h, RNA was isolated, reverse transcribed, and quantitative real-time RT-PCR was performed to quantitate TF expression as described in Materials and Methods (B: blood cells, C: lung).

was not induced (data not shown). In contrast, LPS increased expression of *egr-1* in HUVEC and U937 cells (data not shown). In vivo experiments showed that OxPAPC induced a concentration-dependent increase of *egr-1* in liver and heart (Fig. 3B and C). Interestingly, in liver the induction of *egr-1* by OxPAPC was even stronger than by LPS (Fig. 3B). OxPAPC also induced expression of *egr-1* in lung (data not shown). In aorta and blood cells, *egr-1* expression was weakly induced by LPS but not by OxPAPC (data not shown).

3.4. OxPAPC induces up-regulation of TF in vivo

We have shown previously that OxPAPC induced expression of TF in HUVEC by a mechanism involving EGR-1 (Bochkov et al., 2002). As shown in HUVEC before, U937 cells responded to OxPAPC by increased expression of TF (Fig. 4A). Increased TF expression was observed upon stimulation with OxPAPC in vivo in blood cells and lung (Fig. 4B and C) but not in liver, heart, and aorta (data not shown). LPS increased expression of TF in blood cells (Fig. 4B) but not in lung (Fig. 4C), liver, heart, and aorta (data not shown).

3.5. PAF receptor antagonists block effects of OxPAPC

We have shown previously that PAF receptor antagonists can block the effects of some of the oxidized lipids present in OxPAPC in vitro (Leitinger et al., 1997) and in vivo (Subbanagounder et al., 1999). Here we demonstrate that after pre-injection of CV-3988, a PAF receptor antagonist, the OxPAPC-induced expression of HO-1 in blood cells of mice was blocked (Fig. 5). Injection of CV-3988 alone did not affect the basal expression of HO-1 (Fig. 5).

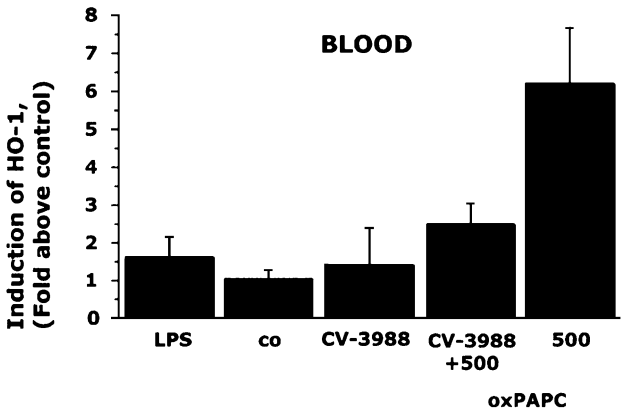

Fig. 5. HO-1 expression in blood cells is blocked by the PAF receptor antagonist CV-3988. Mice were injected with 50 μg of LPS, saline solution (co), or 500 μg of OxPAPC. Mice pretreated with CV-3988 and OxPAPC, received CV-3988 (60 μg) 10 min before injection of OxPAPC (500 μg). Animals were sacrificed after 3 h, blood was collected, and RNA isolated. RNA was then reverse transcribed and quantitative real-time RT-PCR was performed to quantitate HO-1 expression as described in Materials and Methods.

4. Discussion

There is growing evidence that biologically active oxidized phospholipids, which are present in oxidized LDL, oxidized membrane vesicles, or apoptotic cells, induce proinflammatory responses in various cell types in vitro (Berliner et al., 1990; Huber et al., 2002; Watson et al., 1995). However, whether these lipids can exert proinflammatory effects in vivo is unclear, because of the presence of enzymes that can degrade these lipids (Leitinger et al., 1997; Shih et al., 1998; Watson et al., 1995). Thus, we wanted to examine whether after injection of OxPAPC inflammatory genes would be up-regulated in various tissues and whether the patterns of up-regulated genes would differ from those induced by LPS.

Previously, it was shown that endothelial cells and smooth muscle cells respond to MM-LDL by up-regulation of MCP-1 (Cushing et al., 1990). More than 70% of the monocyte chemotactic activity induced by OxPAPC was caused by MCP-1 (Reddy et al., 2001). Using quantitative real-time RT-PCR, we confirmed that MCP-1 expression is increased in endothelial cells upon stimulation with OxPAPC. In our in vivo model, we observed a concentration-dependent increase in JE expression induced by OxPAPC, which was in accordance with our in vitro data. Previously, it has been shown that induction of JE expression in liver can be induced either by intravenous injection of MM-LDL or by feeding mice a high-fat diet (Liao et al., 1991, 1993). These findings suggest that oxidized phospholipids accumulate in the liver and then activate cells to increase JE expression. The fact that we also observed a concentration-dependent increase in JE expression in the heart indicates that oxidized phospholipids were not completely removed by the liver and remains active in plasma.

In order to show a difference in cell activation between OxPAPC and LPS, we compared the expression of E-selectin and HO-1 induced by these agents. E-selectin was up-regulated by LPS but not by OxPAPC in HUVEC. On the other hand, HO-1 was not induced by LPS, but strongly and dose-dependently induced by OxPAPC. In addition, we found that OxPAPC, but not LPS, increased HO-1 expression in U937 cells, demonstrating that monocyte-like cells also specifically respond to OxPAPC. In vivo, we observed similar effects: in all tested tissues E-selectin expression was induced by LPS, but not by OxPAPC, while HO-1 expression was induced by OxPAPC, but not by LPS. These findings suggest that expression of HO-1 in vivo is not regulated via the LPS-induced NFκB pathway but by alternative pathways activated by OxPAPC. HO-1 can serve as a key enzyme in defense against oxidative and cellular stress and expression of inducible HO-1 was shown to be protective in various settings of inflammation (Otterbein and Choi, 2000). Thus, in addition to their proinflammatory effects, oxidized phospholipids may exert anti-inflammatory effects by inducing the up-regulation of HO-1.

EGR-1, which was found in atherosclerotic lesions and which was shown to be a transcription factor for growth factors, hormones, cytokines, lipoproteins, or adhesion molecules (Khachigian and Collins, 1998), also seems to be regulated by oxidized phospholipids. In vitro, we observed a concentration-dependent induction of *egr-1* expression in HUVEC by OxPAPC, whereas in U937 cells OxPAPC did not induce *egr-1* expression. In vivo, we found up-regulation of *egr-1* in liver, heart, and lung. *Egr-1* expression in liver was even higher in OxPAPC-treated mice than in LPS-treated mice. EGR-1 influences the expression of other proteins, like PDGF-A and -B chain, basic fibroblast growth factor, TGF-β, TNF-α, ICAM-1, CD44, p53, urokinase-type plasminogen activator (u-PA) (Khachigian and Collins, 1998), metalloproteinases (Haas et al., 1999), and TF (Mackman, 1995), all of which were shown to be important in vascular disease (Wilcox et al., 1989). Since we demonstrate up-regulation of *egr-1* by oxidized phospholipids in an in vivo model, oxidized lipids may influence inflammatory processes and coagulation by inducing *egr-1*-dependent gene expression.

It has been shown that TF expression is induced by OxPAPC in HUVEC and smooth muscle cells (Bochkov et al., 2002; Drake et al., 1991; Penn et al., 1999, 2000). Here we show for the first time that U937 cells express high levels of TF after stimulation with OxPAPC. In vivo, we illustrate that after injection of OxPAPC TF was up-regulated in blood cells and lung, whereas in liver, heart, and aorta only basal levels of TF were detectable. Thus, oxidized phospholipids may modulate the prothrombotic state of the vascular wall in chronic inflammation.

To examine whether OxPAPC-induced up-regulation of genes was mediated by a receptor, we used PAF receptor antagonists, which had previously been shown to block effects of oxidized phospholipids in vitro and in vivo (Subbanagounder et al., 1999). OxPAPC-induced HO-1 expression in blood cells was blocked by pre-injection of the PAF receptor antagonist CV-3988, suggesting receptor-mediated cell activation by OxPAPC in vivo.

In summary, using the method of quantitative real-time RT-PCR, we were able to demonstrate distinct differences in gene expression in different cell types in vitro and various organs in an in vivo model upon treatment with oxidized phospholipids and LPS. This study illustrates the advantage of using quantitative real-time RT-PCR for analyzing differential regulation of various genes simultaneously and in comparison with different stimuli. Moreover, we conclude that oxidized phospholipids are biologically active in vivo after intravenous injection and that these lipid oxidation products cause systemic effects such as organ-specific up-regulation of different genes and activation of blood cells.

4.1. Future perspectives

The implication that lipid oxidation products such as oxidized phospholipids may promote chronic inflammatory processes certainly opens new possibilities for clinical intervention. Strategies have to be devised in order to modulate the action of lipid oxidation products in vivo. Therefore, it is necessary to elaborate on the mechanism of cell activation by these lipids, to identify specific motifs that are recognized by cells as well as putative cell surface or nuclear receptors, aiming at the synthesis of specific receptor antagonists. Furthermore, for pharmacological intervention on the cellular level, we need to know more about intracellular signaling pathways that are induced by the various lipid oxidation products. Last but not least, gene array techniques will tell us about the genes that are specifically induced by oxidized lipids and discovery of novel genes induced by oxidized lipids will lead to a better understanding of chronic inflammatory diseases.

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