Oxidized Phospholipids Induce Changes in Hepatic Paraoxonase and ApoJ but Not Monocyte Chemoattractant Protein-1 via Interleukin-6*

Received for publication, May 12, 2000, and in revised form, September 15, 2000
Published, JBC Papers in Press, October 16, 2000, DOI 10.1074/jbc.M004074200

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In this study, we tested if interleukin-6 (IL-6) plays a role in mediating the effects of oxidized phospholipids (OXPL). Treatment of HepG2 cells with oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphoryl choline (OX-PAPC), or biologically active lipids present in mildly oxidized low density lipoprotein, increased apoJ mRNA levels. Antibodies to IL-6 blocked these changes. IL-6 treatment in the absence of OXPL produced the same pattern of mRNA changes observed with OXPL treatment alone. In vivo, OX-PAPC injected into C57BL/6J mice resulted in a marked reduction in PON activity and an increase in apoJ levels in plasma after 16 h. Injection of OX-PAPC into IL-6-deficient C57BL/6J mice (IL-6-/−) did not alter either PON activity or apoJ levels. We then tested if other mechanisms involved in fatty streak formation depended upon IL-6. Antibody to IL-6 had no effect on OX-PAPC-induced secretion of MCP-1 by endothelial cells nor on MCP-1 mRNA expression in HepG2 cells. C57BL/6J and IL-6-/− mice fed an atherogenic diet both demonstrated markedly reduced plasma PON activities and the IL-6-/− mice developed fatty streaks to a greater degree than wild-type mice. We conclude that IL-6 is critical to short term but not long term regulation of PON and that IL-6 is not required for OXPL regulation of MCP-1.

We previously reported that high density lipoprotein (HDL)1-associated enzymes can protect LDL against oxidation by aortic wall cells (1, 2). However, we also reported that during an acute phase response (APR) in both humans and rabbits, HDL lost these protective enzymes and was converted from an anti-inflammatory to a pro-inflammatory particle (3). In other studies we observed that feeding an atherogenic diet to mice genetically susceptible to atherosclerosis resulted in changes similar to those seen in the APR. Both the plasma and hepatic expression of two HDL-associated proteins, apolipoprotein (apo) J and paraoxonase (PON), were altered. ApoJ, a marker of the acute phase response, was increased, whereas PON was reduced (4). Previously, we had demonstrated that injection of mildly oxidized LDL into susceptible mice resulted in a similar pattern of expression, i.e. apoJ was increased, whereas PON was reduced (4). More recently we reported that an atherogenic diet when fed to mice induced the hepatic formation of the oxidized phospholipids that are responsible for the biologic activity of mildly oxidized LDL (7).

Feingold and associates (8, 9) observed an increase in hepatic apoJ expression and a decrease in hepatic PON expression in Syrian hamsters with inflammatory cytokines, specifically TNF-α and interleukin (IL)-1. Another principal mediator of the APR, IL-6, is also known to affect the hepatic synthesis of a number of apolipoproteins and acute phase reactants (10), in vivo and in vitro (11), however its role in artery wall metabolism is less well understood. Studies from the literature support both a positive (12) and negative role (13) for IL-6 in the development of atherosclerosis. The data reported here demonstrate that oxidized phospholipids found in mildly oxidized LDL act to acutely alter the expression of PON and apoJ, at least in part, through the inflammatory cytokine, IL-6. However, the mechanisms by which oxidized phospholipids alter PON and apoJ expression in the liver are different from those required to induce MCP-1, and the short-term regulation of PON differs from long term regulation.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture materials and other reagents were obtained from sources described previously (1, 4, 11). PAPC was obtained from Sigma or from Avanti Polar Lipids (Alabaster, AL). Recombinant IL-6, antibodies to IL-6, and ELISA kits to measure murine IL-6 and human MCP-1 levels were obtained from BIOSOURCE (Thousand Oaks, CA).

Cell Culture—HepG2 cells were propagated in 10% fetal bovine serum in Eagle’s modified essential medium supplemented with 1% sodium pyruvate, 1% penicillin-streptomycin-glutamine, 1% nonessential amino acids, and 0.05% Fungizone in tissue culture flasks treated with 0.1–0.5% gelatin at 37 °C overnight. HepG2 cells were seeded at a density of 1 × 10⁵ cells/cm² and allowed to reach a confluence of 80–85% by the time of the experiment.
Human aortic endothelial cells were isolated from donor hearts. Human aortic endothelial cells were subsequently seeded at 2 \times 10^4 cells/cm^2 and were allowed to grow forming a complete monolayer of confluent endothelial cells in 2 days. At the time of experiment, the cells were washed and transferred to Ham’s F-10 medium containing 10% LPDS for 1 h to equilibrate the cells. The cells were subsequently washed, and fresh media containing the experimental additions were added.

Mice—Female C57BL/6J and C57BL/6J mice genetically lacking IL-6 (IL-6−/−), 8–10 weeks of age, were purchased from the Jackson Laboratory, Bar Harbor, ME. Mice were bled under anesthesia by retroorbital puncture, in accordance with protocols approved by the UCLA Animal Research Protection Committee.

Diets—Purina Chow diet (Ralston Purina Co., St. Louis, MO) or an atherogenic diet of 15% fat, 1.25% cholesterol, and 0.5% cholic acid (Teklad, Madison, WI) were used in these studies.

Histological Analysis—The heart, including the aortic root, was dissected out and washed once in phosphate-buffered saline. The basal portion of the heart and aortic root were embedded in OCT compound (Sakura Finetechnical, Kyoto, Japan) and frozen on dry ice. Serial 10-μm-thick cryosections of the heart tissue, covering the area between the appearance of the mitral valves to the disappearance of the aortic valves, were prepared. Every third section was collected on a poly-L-lysine-coated slide. All sections were stained with Oil Red O to assess aortic fatty streak development.

Preparation of Oxidized Lipids—OX-PAPC, 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphorylcholine (m/z 594), and 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphorylcholine (m/z 610), were prepared as described previously (12).

Quantitation of Gene Expression—Total RNA was extracted from the HepG2 cells according to the technique of Chomczynski and Sacchi (13). Northern blot analysis was used to quantitate the mRNA levels of apoJ and PON. For each sample, 15 μg of RNA were electrophoresed on formaldehyde, 1% agarose gels and transferred to 20× SSC equilibrated Hybond ECL™ nitrocellulose membrane. Membranes were hybridized following UV cross-linking and washed at a high stringency (65°C, 0.1× SSC). The apoJ probe had a sequence of: 5′-CAGTCCACAGACAAGATCTCCTGAACTTCTTTCCACTGCG-3′. The PON probe had a sequence of: 5′-ATTACCTCTGTAAGTAGCTAAATCCCATGAGGGTTAATG-3′. The blots were also hybridized using a cDNA probe for 18 S ribosomal RNA, or a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase to normalize the quantities of RNA loaded into the gel lanes.

PON Activity—Plasma samples were assayed for PON activity using paraaxon as substrate (15). The cuvette contained 1.0 m M paraoxon in 20 mM Tris-HCl, pH 8.0. The reaction was initiated by the addition of the plasma sample and the increase in the absorbance at 405 nm was measured. Enzymatic activity was calculated according to the equation: 

\[ \text{Activity} = \frac{A_{405}}{A_{405}^0} \times \text{S.D. of density scans} \times \text{dilution factor} \times \text{volume} \]

with respect to apoJ mRNA expression, when the cells were treated with PAPC, treatment of cells with the oxidized phospholipids having a m/z of 594 or 610. Total RNA was prepared for Northern blot analysis as described under “Experimental Procedures.” Values shown are the mean ± S.D. of densitometric scans from three separate experiments normalized for 28 S ribosomal RNA expression. Asterisks indicate significant difference at the level of p < 0.05, between medium alone and each treatment.

RESULTS

Effects of Oxidized Lipids on PON and ApoJ mRNA Expression in HepG2 Cells—In previous studies we had observed that HepG2 cells exposed to LDL that had been oxidized by copper ions of smooth muscle cells and endothelial cells (CM-LDL) for 16 h demonstrated a decrease in mRNA for PON (16) and an increase in apoJ mRNA (4). To determine whether specific oxidized arachidonic acid-containing phospholipids would produce similar results, the experiments shown in Figs. 1 and 2 were carried out. HepG2 cells incubated for 16 h in medium alone or in medium containing 100 μg/ml nonoxidized phospholipid PAPC showed no differences with respect to the levels of expression of PON mRNA (Fig. 1). However, in HepG2 cells exposed to either 100 μg/ml OX-PAPC or 10 μg/ml amounts of two synthetic oxidized phospholipids having m/z of 594 and 610, respectively (16), a marked decrease in PON mRNA expression was observed. In addition, as demonstrated in Fig. 2, whereas no significant differences were seen in HepG2 cells with respect to apoJ mRNA expression, when the cells were treated with PAPC, treatment of cells with the oxidized phos-
Oxidized Lipids and IL-6

Role of IL-6 in Mediating the Effects of Oxidized Phospholipids on HepG2 Cells—One of the principal mediators of both acute and chronic inflammatory responses in many diseases is IL-6. To test if this cytokine might play a role in the inflammatory responses elicited by the oxidized phospholipids, the experiment shown in Fig. 3 was carried out. HepG2 cells were incubated in tissue culture medium alone, in medium containing 100 μg/ml Ox-PAPC, in medium containing 1 μg/ml neutralizing antibody to IL-6 alone, or in medium containing 100 μg/ml Ox-PAPC but also containing 1 μg/ml neutralizing antibody to IL-6. Fig. 3 illustrates that, similar to what was seen in Fig. 2, Ox-PAPC inhibited the expression of PON mRNA in HepG2 cells. However, in the presence of neutralizing antibody to IL-6, there was a marked blunting of the decrease in PON mRNA expression in response to Ox-PAPC. Likewise, as shown in Fig. 3B, neutralizing antibodies to IL-6 blocked the stimulation of apoJ mRNA by Ox-PAPC. Medium containing neutralizing antibodies to IL-6 had no significant effect on either PON or apoJ mRNA expression. The differences in apoJ or PON mRNA in media alone, α-IL-6 in media, or Ox + α-IL-6 were not statistically significant. Moreover, irrelevant antibodies had no effect on either PON or apoJ mRNA expression (data not shown). To directly test the effects of IL-6, the experiment in Fig. 4 was conducted. HepG2 cells were incubated in medium alone, or in medium containing a dose of recombinant IL-6 shown to be active in vitro (100 ng/ml) (11) for 16 h and then mRNA levels for PON and apoJ measured. IL-6 was effective in mimicking the effects of Ox-PAPC on PON and apoJ mRNA expression, causing a reduction in PON mRNA and a stimulation of apoJ mRNA.

Absence of IL-6 Results in an Altered Response to Oxidized Lipids—We had previously demonstrated that injection of Ox-PAPC into C57BL/6J mice resulted in a marked reduction in plasma PON activity (4). If IL-6 has a role in mediating the effects of oxidized lipids on altering apoJ levels and PON activity, then one might predict that, in the absence of IL-6, this response would be diminished. We therefore used IL-6 knockout mice (IL-6 −/−) bred onto a C57BL/6J background to test the effects of Ox-PAPC on altering plasma apoJ levels and PON activity in these mice. Fig. 5A shows that, compared with un.injected mice or mice receiving an intravenous injection of saline or PAPC, injection of Ox-PAPC into the wild type strain, C57BL/6J mice (WT) resulted in a marked decrease in plasma PON activity. Moreover, this same concentration of Ox-PAPC resulted in an increase in plasma apoJ levels (Fig. 5B). When plasma levels of IL-6 were measured by ELISA in WT mice 16 h after Ox-PAPC injection, they were found to be elevated 5-fold compared with saline or PAPC-treated mice (data not shown). In contrast, neither plasma apoJ levels nor plasma PON activity was altered in IL-6 −/− mice after injection of Ox-PAPC compared with plasma PON activities in IL-6 −/− mice that were either untreated, or treated with saline or PAPC. Both strains, however, responded to an injection of LPS, demonstrating an increase in apoJ and a reduction in PON activity. Injecting a lower dose of 2.5 μg of LPS resulted in similar but smaller changes in both apoJ and PON activity (data not shown). Expression of mRNA for both PON and apoJ reflected the plasma PON profiles in the mice (data not shown). These results would suggest that an IL-6-independent pathway is involved in the action of LPS on apoJ and PON.

Since IL-6 −/− mice did not exhibit the Ox-PAPC-induced reduction in plasma PON activity observed in C57BL/6J mice, we tested the hypothesis that IL-6 may have a role in modulating the aortic response to oxidized phospholipids. To first address this question, we examined the effects of Ox-PAPC on the production of a key chemokine by endothelial cells, MCP-1, in the absence of IL-6, by using neutralizing antibody to this cytokine. As shown in Fig. 6, compared with endothelial cells incubated in the presence of PAPC, the addition of Ox-PAPC resulted in an increase in MCP-1 in the medium similar to the levels seen with LPS and IL-6 alone. However, neutralizing antibody to IL-6, which reduced the level of MCP-1 induced by IL-6 alone (data not shown), had no significant effect on the induction of MCP-1 by Ox-PAPC. This raised two questions. First, is the role of IL-6 in the response to oxidized phospholipids unique to the liver cell, and second, are the mechanisms responsible for inducing apoJ and PON different from those for MCP-1? Fig. 7 shows that, in contrast to the effects of antibody to IL-6 on Ox-PAPC-mediated alterations in PON and apoJ in HepG2 cells (Fig. 3), antibody to IL-6 had no effect on Ox-PAPC-mediated MCP-1 expression in HepG2 cells. This would suggest that, indeed, the mechanisms mediating the action of oxidized phospholipids on PON and apoJ are different from those required to induce MCP-1.

These results suggested that, acutely, IL-6 plays a role in...
PON regulation. However, would IL-6 also play a role in the regulation of PON as well as in fatty streak development in the face of a long term oxidative stress? To test this, we fed WT and IL-6/2/2 mice an atherogenic diet for 15 weeks and then measured both plasma PON activity and liver mRNA levels as well as the extent of aortic fatty streak formation. As shown in Fig. 8A, PON activity decreased in both WT and IL-6/2/2 fed the atherogenic diet, compared with mice on chow. Changes in PON mRNA essentially mirrored changes in activity (Fig. 8B). The extent to which the PON activity decreased in the IL-6/2/2 on the atherogenic diet was less than in the WT; however, these differences were small and there was a greater than 80% reduction in both groups of animals. The small differences in activity could not be detected as differences in mRNA expression between WT and IL-6/2/2. Despite similar low PON activity and mRNA levels, IL-6/2/2 mice were found to have significantly greater areas of fatty streak involvement in their aortas than did WT mice (Fig. 9). Neither strain developed lesions on a chow diet (data not shown). These results would suggest that, under the conditions employed here (the atherogenic diet), relatively small changes in PON activity were overshadowed by other events in the artery wall.

**DISCUSSION**

Our previous results in animal models demonstrated that the decrease in the PON/apoJ ratio induced by administration of oxidized phospholipids, by a high fat atherogenic diet, or during an APR contributed to the formation of HDL that were no longer anti-inflammatory in nature but were in fact pro-inflammatory (3, 4). In the present study, we have demonstrated that specific oxidized phospholipids found in MM-LDL affected the gene expression of two HDL-associated proteins, PON and apoJ, when incubated with cultured HepG2 cells. This response was blocked by antibodies to IL-6. HepG2 cells treated directly with IL-6 in the absence of oxidized phospholipids showed the same pattern of response for PON and apoJ mRNA expression.

Others have noted effects of cytokines on hepatic lipoprotein synthesis, suggesting a link between the acute phase response and the development of atherosclerosis. Memon and co-workers (14) observed that both IL-1 and tumor necrosis factor increased serine palmitoyltransferase, the rate-limiting step in sphingolipid synthesis, resulting in an altered lipoprotein structure. Song et al. (15) observed that IL-1β and TNF-α suppressed apoA-I expression in HepG2 cells but not expression of apoE. Feingold and co-workers (9) demonstrated that HepG2 cells treated with the other major APR cytokines, TNF-α and IL-1, showed a decrease in PON mRNA levels. Moreover, other changes in lipoprotein metabolism that could potentially influence the development of atherosclerosis are increases in serum triglyceride levels, an increase in small dense LDL, changes in the composition of HDL, including the activities of its enzymes other than PON, particularly lecithin cholesterol acyltransferase and cholesteryl ester transfer protein (16, 17).

We previously demonstrated that PON is capable of abolishing the bioactivity of mildly oxidized LDL and that of OX-PAPC (1). Alterations in plasma PON activity have been reported in several pathological conditions. Abbott and colleagues (18) and Mackness and Durrington (19) have reported low PON activity in diabetes and that a low serum PON activity was associated with increased susceptibility to atherosclerosis (20). ApoJ, also known as clusterin, is a glycoprotein that is present in a sub-
population of HDL together with PON and apoA-I (21, 22). It has been reported to have roles in complement regulation and prevention of cytolysis (23), lipid transport (24), apoptosis (24), and membrane protection at fluid-tissue interfaces where it is expressed (25). HepG2 cells secrete apoJ as a lipoprotein, indicating its ability to be associated with lipid (26). One could postulate that the association of apoJ and PON in the plasma, and their reciprocal regulation by the liver, might act as a coordinated protection during inflammatory conditions.

These studies support the notion that, during an acute oxidative stress, the effects of oxidized phospholipids may be mediated by inflammatory cytokines. The action of IL-6 may be via its activation of gp130, a transmembrane glycoprotein involved in many cytokine-mediated cellular responses (27). Additional support for a role of oxidant stress on cytokine activation comes from the work of Eugui et al. (28). These investigators found that antioxidants such as butylated hydroxyanisole, tetrahydropapaveroline, nordihydroguaiaretic acid, and 10,11-dihydroxyaporphine inhibited the production of IL-6 as well as TNF-α and IL-1β by human peripheral blood monocytes, suggesting that suppressing oxidation can limit the inflammatory response.

In the present study, injection of OX-PAPC into C57BL/6J mice resulted in a dramatic reduction in plasma PON activity after 16 h. However, injection of OX-PAPC into IL-6−/− mice had no such effect. Others have found that, in IL-6-deficient mice, IL-6 was absolutely required for the transcriptional induction of hepatic APR genes following injection of turpentine oil (29).

Tissue levels of oxidized phospholipids were not measured in the in vivo studies reported here. Therefore, we cannot determine the role of oxidized lipids in vivo directly from these studies. For the purpose of discussion, we assume that these lipids were formed as they were in our previous studies (7). To determine whether the cells of the artery wall behave differently with respect to IL-6 in response to oxidized phospholipids, we incubated aortic endothelial cells with OX-PAPC in the presence or absence of neutralizing antibody to IL-6 and measured the release of MCP-1 into the tissue culture medium. We had previously demonstrated, using neutralizing antibody to MCP-1, that >95% of the monocyte chemotactic activity in an artery wall coculture system was due to the presence of MCP-1 (30). Although IL-6 itself increased MCP-1 secretion from endothelial cells, we found that neutralizing antibody to IL-6 did not alter OX-PAPC-induced MCP-1 secretion by endothelial cells (Fig. 6). Moreover, neutralizing antibody to IL-6 had no effect on OX-PAPC-induced MCP-1 mRNA expression in HepG2 cells (Fig. 7). We reasoned that if indeed IL-6 had a role in mediating the action of oxidized lipids in the liver, then this cytokine might also have an influence on the events in the artery wall leading to the development of fatty streaks. To test
this hypothesis, IL-6 /−/− mice were fed an atherogenic diet for 15 weeks and compared with their background strain, C57BL/6J mice, for the extent of lesion development. To our surprise, IL-6 /−/− mice were found to have significantly greater areas of fatty streak involvement in their aortas than did WT mice (Fig. 9) despite the small but significant changes
in PON activity. Sukovich (13) recently reported in abstract form that there was no difference in plaque surface area in apoE/IL-6 double knockout mice compared with apoE /−/− mice, but that the plaques were less fibrotic. IL-6 /−/− mice treated with lipopolysaccharide had a 3-fold greater production of TNF-α than in wild type controls (31). In other studies, IL-6 /−/− mice had 30–50% of the circulating plasma levels of TNF-α and IL-1β (32). In our study, the decrease in PON activity in IL-6 /−/− mice was slightly although significantly less than in the WT mice on the atherogenic diet. However, there was a substantial reduction in PON activity in both the IL-6 /−/− and the WT mice fed the atherogenic diet compared with the mice fed a chow diet. The difference between the results in Figs. 5 and 8 may result from a difference in the magnitude and duration of the oxidative stress (15 weeks on the atherogenic diet). In the chronic situation, PON activity was decreased despite the absence of IL-6. This suggests that IL-6 may be critical to short term regulation of PON, but not to long term regulation. If these results can be extrapolated to humans, it might also suggest that short term regulation of PON activity mediated by IL-6, together with the IL-6 induction of MCP-1 in endothelial cells (Fig. 6), might exacerbate the inflammatory reaction in an established atherosclerotic lesion.

A number of epidemiological studies have observed a link between acute inflammation and cardiovascular diseases. Changes in the levels of plasma C-reactive protein have been linked with an increased cardiovascular risk (33). Concentrations of C-reactive protein and serum amyloid A have also been shown to increase acutely after cholecystectomy, returning to normal within 2 weeks (34). Plasma concentrations of IL-6 are elevated in a number of inflammatory states as diverse as rheumatoid arthritis (35), lymphoma (36), orthopedic surgery (37), and cardiovascular disease (38). Since IL-6 has been shown to induce the serum amyloid A promoter (39), it is possible that factors that can elevate IL-6 levels, such as oxidized phospholipids, may be an initial step in the alterations observed during an acute phase response. In mice, inoculation with murine cytomegalovirus increased LDL-derived cholesterol and resulted in a greater incidence of early atherosclerotic lesions (40). *Chlamydia pneumoniae* has been repeatedly demonstrated to be associated with the incidence of atherosclerosis by both serology and the demonstration of the organism in atherosclerotic lesions (41). An intriguing relationship has also been made between acute respiratory infections and the risk of first time acute myocardial infarction (42). Another study has shown that the presence of an inflammatory response has a prognostic value in patients with unstable angina and may predict the long term risk of cardiovascular events (43). Finally, there are results of two preliminary antibiotic treatment trials using azithromycin for the secondary prevention of cardiovascular disease (44). It is possible that the nonspecific immune response to these infectious agents, the APR, may play a role, and may be a mechanism linking infections with chronic inflammatory processes. Work in this laboratory is currently focused on the specific initiators of the oxidant stress-mediated development of early atherosclerotic lesions, and investigating further the relationship between lipid oxidation and the APR.

Acknowledgments—We are thankful to members of the heart transplant team for providing the donor aortic specimens for isolation of the aortic endothelial cells. We also thank Susan Hama, Rachel Mottahedeh, and Linda Jin for expert technical assistance.

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J. Biol. Chem. 2001, 276:1923-1929.
doi: 10.1074/jbc.M004074200 originally published online October 16, 2000

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