High-Density Lipoprotein Modulates Oxidized Phospholipid Signaling in Human Endothelial Cells From Proinflammatory to Anti-inflammatory

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Objective—Oxidized 1-palmitoyl-2-arachidonyl-sn-3-glycero-phosphorylcholine (Ox-PAPC) and its component phospholipid, 1-palmitoyl-2-(5,6 epoxyisoprostanoyl)-sn-glycero-3-phosphocholine (PEIPC), which are present in atherosclerotic lesions, activate endothelial cells to induce a complex inflammatory and pro-oxidant response. Previously, we demonstrated induction of genes regulating chemotaxis, sterol biosynthesis, the unfolded protein response, and redox homeostasis by Ox-PAPC in human aortic endothelial cells (HAECs). Activation of the c-Src kinase/signal transducer and activator of transcription 3 and the endothelial nitric oxide synthase/sterol regulatory element binding protein (SREBP) pathways were shown to regulate several of these inflammatory effects of Ox-PAPC in HAECs. The goal of the current studies was to determine the role of high-density lipoprotein (HDL) in regulating Ox-PAPC signaling in HAECs.

Methods and Results—Using quantitative real-time polymerase chain reaction, Western analysis, and functional studies, we demonstrated that pretreatment of HAECs with HDL reduced the induction of inflammatory, sterol biosynthetic, and unfolded protein response genes by Ox-PAPC and PEIPC; Ox-PAPC-induced chemotactic activity and monocyte binding were also decreased. These effects were associated with HDL inhibition of Ox-PAPC-induced c-Src, signal transducer and activator of transcription 3, and SREBP activation, alterations in endothelial nitric oxide synthase phosphorylation (previously associated with the inflammatory action of Ox-PAPC), and a decrease in superoxide formation. Finally, we demonstrated that treatment with HDL did not inhibit Ox-PAPC and PEIPC-induced activation of redox pathways, which protect the cell from the effects of oxidative stress.

Conclusions—Taken together, these studies demonstrated that HDL inhibits the pro-inflammatory effects of Ox-PAPC and PEIPC, while maintaining the antioxidant activities of these lipids. (Arterioscler Thromb Vasc Biol. 2007;27:1346-1353.)

Key Words: atherosclerosis ■ endothelium ■ inflammation ■ oxidized phospholipids

Atherosclerosis is a chronic inflammatory condition characterized by lipid accumulation and monocyte entry into the subendothelium. Phospholipid oxidation products of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (PAPC), present in mildly oxidized low-density lipoprotein (LDL) and accumulates in sites of chronic inflammation, including atherosclerotic lesions, activate endothelial cells (ECs), leading to enhanced monocyte/EC interactions.1 The importance of oxidized phospholipids in atherosclerosis is also suggested by the observation that levels of enzymes that increase oxidized phospholipids, such as myeloperoxidase, are an indicator of increased atherosclerosis;2,3 furthermore, polymorphisms that result in loss of function for enzymes that degrade oxidized phospholipids also are associated with increased atherosclerosis.1 An important study by Tsimikas et al3 demonstrates the presence of oxidized phospholipids in human plasma associated with apo(a) and has shown them to be predictive of the presence and progression of atherosclerosis. Previously we reported that treatment of human aortic ECs (HAECs) in culture with oxidized PAPC (Ox-PAPC) and its most active component phospholipid, 1-palmitoyl-2-epoxyisoprostane E2-sn-glycero-3-phosphocholine (PEIPC), induces a complex response characterized by the activation of several molecular pathways and the regulation of ~1000 genes.4 Many of these genes were also shown to be induced in aorta of mice after injection of Ox-PAPC.5 We and others have demonstrated induction of genes regulating chemotaxis,6,7 sterol metabolic,8 the unfolded protein response,4 thrombosis,10 and redox homeostasis5 by Ox-PAPC and mildly oxidized LDL treatment of HAECs. Our group also
demonstrated that activation of the c-Src kinase/signal transducer and activator of transcription (STAT) 3,11 the endothelial nitric oxide synthase (eNOS)/sterol regulatory element binding protein (SREBP) pathway,9 and the unfolded protein response (UPR) pathways12 regulated the levels of the inflammatory genes, monocyte chemoattractant protein (MCP)-1, IL-6, and IL-8 in HAECs. Furthermore, the eNOS/SREBP pathway regulated the induction of cholesterol metabolic genes, LDL receptor, and Insig1 by Ox-PAPC and PEIPC. Activated SREBP8 and UPR proteins activating transcription factor (ATF) 3 and ATF412 were also shown to be increased in the inflammatory areas of human atherosclerotic lesions, but not in areas that lacked inflammatory cells. These lesion areas also costained with E06 antibody, which detects oxidized phospholipids, suggesting that the pathways identified in vitro are also activated in vivo. All of these data suggest an important role for oxidized phospholipids in atherosclerotic lesion formation and other chronic inflammatory processes.

The goal of the current studies was to determine the effect of high-density lipoprotein (HDL) on the activation of ECs by Ox-PAPC and its component PEIPC. Extensive epidemiological, genetic and clinical data support the role of HDL as a protective factor against atherogenesis and coronary heart disease.13 While “reverse cholesterol transport” is currently the most widely accepted mechanism by which HDL prevents atherosclerosis, HDL has other properties that could mediate its protective effects.14 Several lines of evidence suggest that HDL possesses potent anti-inflammatory and antioxidant properties15–17. There is growing evidence that HDL and its mimetics inhibit inflammation and endothelial dysfunction by inhibiting the oxidative stress produced by hypercholesterolemia resulting in increased level of NO.18,19 To gain further insight into the atheroprotective effects of HDL, we examined the effect of HDL on the pro-inflammatory and pro-oxidant properties induced by Ox-PAPC.

In the current studies, we demonstrate that pretreatment of HAECs with HDL reduced Ox-PAPC–induced activation of inflammatory signaling pathways and superoxide production; HDL pretreatment, however, preserved the induction of signaling pathways protecting against inflammation and oxidative stress by Ox-PAPC. Taken together, our findings demonstrate a protective role for HDL in regulating oxidized phospholipid signaling in human ECs.

**Materials and Methods**

**Reagents**

M199 medium for HAECs was purchased from Irvine Scientific. Fetal bovine serum was obtained from HyClone. PAPC was purchased from Avanti Polar Lipids. Oxidized phospholipids were prepared as described previously.20 Rabbit polyclonal phosphospecific antibodies against c-Src kinase Y418, STAT3 Y705, eNOS S1177, eNOS T495, total STAT3, and histone H1A were purchased from Cell Signaling. Rabbit polyclonal antibodies against SREBP, ATF3, HO-1, NRF2 total eNOS, total c-Src kinase, and GAPDH were purchased from Santa Cruz Biotechnology. HDL was obtained from the Atherosclerosis Research Unit Core facilities and isolated using sequential ultracentrifugation (1.063 < d <1.21).

**Cell Culture, Chemotaxis, and Monocyte Binding**

HAECs were isolated from the aortic rings of explanted donor hearts and cultured as previously described.9 HMEC, obtained from the Center for Disease Control, was cultured as previously described.21 Treatment with lipids and other activating agents was performed in media supplemented with 1% to 2% (vol/vol) fetal bovine serum. The monocyte binding assays were performed as described previously by our group22 using isolated monocytes from normal human donors. The monocyte chemotaxis assay in response to medium isolated from cells treated with oxidized lipid or oxidized lipid and HDL were performed as described previously using monocytes from normal human donors and chemotaxis measurements in a neuroprobe chamber.22

**Cell Lysate Preparation**

Confluent endothelial cells (HAECs and HMECs) were harvested and lysed in radioimmunoprecipitation assay buffer. After centrifugation at 5000 rpm for 10 minutes, the supernatants (cell lysates) were collected. Nuclear extracts were prepared as previously described.9

**Quantitative Real-Time Polymerase Chain Reaction Using SYBR Green Chemistry**

All primers were designed using Integrated DNA Technologies’ Primer Quest online design tool. Quantitative real-time polymerase chain reaction was performed and data were analyzed as previously described. The primers used were also included in this article.9

**Measurement of Superoxide Production**

Intracellular superoxide production was determined using oxidation of NBT.23 Cells were pretreated for 1 hour with or without HDL in medium containing 1% serum. Then cells were rinsed and medium containing 400 μg/mL NBT with or without Ox-PAPC (and with or without HDL) and incubated for an additional 1 hour.

**Results**

**HDL Inhibits Ox-PAPC-induced Inflammatory, Sterol Biosynthetic, and UPR Gene Expression, As Well As Chemotactic Activity and Monocyte Binding Induced by Ox-PAPC**

To determine the effect of HDL on Ox-PAPC signaling, we first examined the effect of HDL on the regulation of inflammatory pathways. Our data demonstrated that pretreatment of HAECs with HDL (100 μg/mL) for 1 hour, followed by cotreatment, significantly reduced the induction of chemotactic genes IL-8, MCP-1, and IL-6 mRNA by Ox-PAPC (Figure 1A). HDL (100 μg/mL) also significantly reduced the induction of IL-8 protein expression by Ox-PAPC, as measured by enzyme-linked immunosorbent assay (Figure 1B). We previously demonstrated that IL-8 regulates Ox-PAPC–induced monocyte binding, whereas MCP-1 is the main regulator of chemotactic activity induced by Ox-PAPC. In these studies, both Ox-PAPC–induced monocyte binding and chemotactic activity were inhibited by HDL pretreatment (Figure 1C). HDL (100 μg/mL) pretreatment also significantly reduced the induction of sterol biosynthetic genes, LDL receptor, Insig-1, and 3-hydroxy-3-methylglutaryl-coenzyme A synthase by Ox-PAPC (Figure 1D). Furthermore, HDL significantly reduced the induction of IL-8 mRNA (Figure 1E, left panel) and LDL receptor mRNA (Figure 1E, right panel) by PEIPC. These findings demonstrated that Ox-PAPC–induced (and PEIPC-induced) chemotactic and sterol biosynthetic gene expression by HDL.

We also examined the protective role of HDL in regulating the induction of UPR genes, ATF-3, ATF-4, and spliced...
HAECs were either untreated (control) or treated for 4 hours with Ox-PAPC (50 μg/mL). IL-8 mRNA (left panel) and LDL receptor mRNA (right panel) were analyzed using quantitative real-time polymerase chain reaction. E, LDL receptor, Insig-1, and 3-hydroxy-3-methylglutaryl-coenzyme A synthase mRNA levels were also analyzed using quantitative real-time polymerase chain reaction. A to E, Individual treatments were performed in triplicate; data represent mean ± SD and *P<0.05 indicates significance (comparison between Ox+HDL and Ox groups). All experiments were repeated at least 3 times.

Figure 1. HDL inhibits the induction of chemotactic and sterol biosynthetic genes, as well as monocyte binding and chemotactic activity induced by oxidized phospholipids. HAECs were either untreated (control) or treated for 4 hours with Ox-PAPC (50 μg/mL). IL-8, MCP-1, and IL-6 mRNA levels were analyzed using quantitative real-time polymerase chain reaction. B, IL-8 protein expression was analyzed using enzyme-linked immunosorbent assay. C, Cells were also analyzed for monocyte binding and for monocyte chemotactic activity. D, LDL receptor, Insig-1, and 3-hydroxy-3-methylglutaryl-coenzyme A synthase mRNA levels were also analyzed using quantitative real-time polymerase chain reaction. E, HAECs were either untreated (control) or treated for 4 hours with PEIPC (1 μg/mL) in the presence and absence of human HDL (100 μg/mL). IL-8 mRNA (left panel) and LDL receptor mRNA (right panel) were analyzed using quantitative real-time polymerase chain reaction. A to E, Individual treatments were performed in triplicate; data represent mean ± SD and *P<0.05 indicates significance (comparison between Ox+HDL and Ox groups). All experiments were repeated at least 3 times.

XBP-1 by Ox-PAPC and PEIPC. Our results demonstrated that HDL (100 μg/mL) significantly reduced the induction of ATF-3, ATF-4, and spliced XBP-1 mRNA by Ox-PAPC as measured using quantitative real-time polymerase chain reaction (Figure 2A, left and right panels). Using Western analysis, HDL also significantly reduced the induction of ATF3 (Figure 2B) and ATF4 protein (data not shown) by Ox-PAPC. Furthermore, HDL significantly decreased the induction of ATF3 mRNA by PEIPC (Figure 2C). These findings demonstrated a protective role for HDL in regulating the activation of the UPR pathway by Ox-PAPC and PEIPC.

In comparison to HDL pretreatment, we observed that pretreatment with LDL was less effective at inhibiting IL-8 and MCP-1 induction by Ox-PAPC (% inhibition of Ox-PAPC–induced IL-8 mRNA levels: HDL = 69 ± 4, LDL = 29 ± 3), but was equally effective at reducing LDL receptor induction by Ox-PAPC (data not shown); LDL, in comparison to HDL, had no effect on reducing the induction of ATF3, ATF4, or XBP-1 by Ox-PAPC (data not shown).

HDL Inhibits or Alters Inflammatory and Sterol Regulatory Pathways Activated by Ox-PAPC
To determine the mechanism by which HDL differentially regulated Ox-PAPC signaling, we next examined the effect of HDL on various signaling molecules activated by Ox-PAPC. Previously, we had demonstrated a role for the c-Src kinase/STAT3 pathway in the early induction of IL-8 transcription by Ox-PAPC.11 We had reported that the sustained induction of IL-8 transcription by Ox-PAPC was regulated through an alternative pathway involving eNOS and SREBP.89 Therefore, we examined the effect of HDL on the activation of these pathways by Ox-PAPC using Western analysis. Treatment of HAEC with HDL (100 μg/mL) significantly reduced Ox-PAPC–induced phosphorylation of c-Src kinase at Y418, a marker of c-Src activation (Figure 3A, top panel); HDL had no significant effect on total c-Src levels (Figure 3A, bottom panel). HDL also significantly reduced the phosphorylation of STAT3 at Y705, a marker of STAT3 activation, by Ox-PAPC (Figure 3B, top panel), but had no effects on total STAT3 expression (Figure 3B, bottom panel). We next examined the effect of HDL on the abundance of the immature and mature forms of SREBP after Ox-PAPC treatment. Our findings demonstrated that HDL significantly inhibited the activation of SREBP by Ox-PAPC, as depicted by an increase in the 125-kDa isoform, and a decrease in the 68kDa isoform (Figure 3C, top panel).

We had previously demonstrated that the activation of SREBP by Ox-PAPC was regulated by eNOS and that Ox-PAPC induced eNOS phosphorylation at serine 1177 and dephosphorylation at T496 (Figure 3D, top panel); total eNOS levels (Figure 3D, bottom panel). Ox-PAPC–induced phosphorylation of eNOS at Y418, a marker of eNOS activation (Figure 3E, top panel); total eNOS kinase activity (Figure 3E, bottom panel). Ox-PAPC–induced phosphorylation of eNOS at Y418, a marker of eNOS activation (Figure 3F, top panel); total eNOS kinase activity (Figure 3F, bottom panel). Ox-PAPC–induced phosphorylation of eNOS at Y418, a marker of eNOS activation (Figure 3G, top panel); total eNOS kinase activity (Figure 3G, bottom panel). Ox-PAPC–induced phosphorylation of eNOS at Y418, a marker of eNOS activation (Figure 3H, top panel); total eNOS kinase activity (Figure 3H, bottom panel). Ox-PAPC–induced phosphorylation of eNOS at Y418, a marker of eNOS activation (Figure 3I, top panel); total eNOS kinase activity (Figure 3I, bottom panel).

We had previously demonstrated that the activation of SREBP by Ox-PAPC was regulated by eNOS and that Ox-PAPC induced eNOS phosphorylation at serine 1177 and dephosphorylation at T496; this phosphorylation pattern was associated with both the activation of eNOS (as measured by increased conversion of arginine to citrulline and the production of superoxide through eNOS).9 Superoxide generation by this enzyme was necessary for SREBP activation. In the current studies, our data demonstrated that HDL had no effect on Ox-PAPC–induced eNOS S1177 phosphorylation; treatment of HAECs with HDL alone for 5 hours also had no effect on eNOS phosphorylation (Figure 3D, top panel). HDL, however, completely reversed Ox-PAPC–induced eNOS T dephosphorylation (Figure 3E, top panel); total eNOS expression remained unchanged (Figure 3D, 3E, bottom panel). This change in eNOS phosphorylation may also contribute to the decrease in SREBP activation. A potential mechanism by which
HDL might inhibit some of these signaling pathways is by inhibition of the formation of reactive oxygen species, which we9 and others24 have previously reported to be increased by Ox-PAPC. Therefore, we examined the effect of HDL on Ox-PAPC–induced superoxide production using an NBT assay for intracellular superoxide. Pretreatment with HDL alone only marginally decreased the levels of superoxide produced in untreated cells; Ox-PAPC–induced superoxide production, however, was reduced by 50% with HDL treatment (Figure 3F). Taken together, these studies demonstrate that HDL downregulates several major pathways regulating inflammatory and sterol metabolic gene expression by Ox-PAPC. A mechanism of this downregulation may involve a general decrease in formation of reactive oxygen species.

**HDL Does Not Inhibit the Induction of Redox Genes by Ox-PAPC**

We next examined the effect of HDL on the induction of redox genes by Ox-PAPC. Using quantitative real-time polymerase chain reaction, Ox-PAPC inductions of heme oxygenase (HO)-1 and heat-shock protein A1A mRNA were analyzed in the presence and absence of human HDL (100 μg/mL). Our results demonstrated that HDL (100 μg/mL) did not reduce the induction of HO-1 (Figure 4A, left panel) or heat-shock protein A1A (Figure 4A, right panel) by Ox-PAPC. There was also no inhibition of the induction of thioredoxin reductase by Ox-PAPC (data not shown). HDL was also ineffective at inhibiting the induction of HO-1 protein expression by Ox-PAPC, as measured by Western analysis (Figure 4B). Furthermore, our data demonstrated that HDL did not inhibit the induction of HO-1 mRNA by PEIPC (Figure 4C). These findings demonstrated that HDL, while inhibiting the induction of inflammatory, sterol biosynthetic, and UPR genes by Ox-PAPC and its component PEIPC, did not inhibit the induction of redox genes by these oxidized phospholipids.

**Ox-PAPC Induced Nrf2 Nuclear Translocation and HDL Does Not Inhibit This Increase**

Because HDL was ineffective at reducing the induction of HO-1 (and heat-shock protein A1A) expression by Ox-PAPC, we examined the effect of HDL on nuclear levels of Nrf2, a known transcriptional regulator of HO-1.25,26 Nrf2, in its inactive state, is localized in the cytoplasm, repressed by its association with the chaperone, Keap; on activation, it disso-
activates an HO-1 promoter reporter construct containing 2 antioxidant response elements. We therefore examined whether Ox-PAPC treatment would activate Nrf2. Western analysis of nuclear lysates obtained from HAECs treated with Ox-PAPC demonstrated that Ox-PAPC treatment increased Nrf2 nuclear translocation within 1 hour and for at least 4 hours after treatment (Figure 5A, top panel). The increase in nuclear levels of Nrf2 likely represents Nrf2 activation, because levels of Nrf2 mRNA remained unchanged after treatment (data not shown). Next, we examined whether HDL would inhibit Nrf2 activation by Ox-PAPC. Our data demonstrated that pretreatment with HDL did not reduce the nuclear levels of Nrf2 induced by Ox-PAPC (Figure 5B, top panel). These findings suggest that HDL was ineffective at reducing HO-1 expression by Ox-PAPC, because HDL did not inhibit Ox-PAPC–induced Nrf2 activation.

Discussion

The results of our current studies extend previous in vivo findings showing that HDL inhibits inflammation and oxidative stress.15,16 We demonstrate that HDL treatment of HAECs downregulates the inflammatory and pro-oxidant effects of Ox-PAPC and its component PEIPC, which accumulate in atherosclerotic lesions. Pretreatment of HAECs with HDL for 1 hour, followed by cotreatment, significantly reduced the induction of chemotactic (Figure 1A), sterol biosynthetic (Figure 1A), and UPR genes (Figure 2A) by Ox-PAPC, respectively; in the present studies, HDL was shown to reduce both of these inflammatory functions (Figure 1D). The monocyte-binding study was also confirmed in medium containing 10% LPDS, in which HDL still demonstrated significant inhibition of Ox-PAPC–induced monocyte binding (data not shown). HDL was significantly less effective at inhibiting Ox-PAPC signaling when presented to HAECs for a 1-hour pretreatment alone, in the absence of coincubation (data not shown). One potential explanation for the requirement for coinubation of HDL with Ox-PAPC could be that HDL contains enzymes that can degrade Ox-PAPC.16 We have obtained evidence that HDL (100 μg/mL) degrades 40% of POVPC and 30% of PEIPC after a 30-minute coincubation with these oxidized phospholipids (data not shown). In the current studies, however, HDL did not inhibit the induction of redox genes (Figure 4) or the phosphorylation of eNOS at serine 1177 (Figure 3D) by Ox-PAPC, indicating that the phospholipid stimulating these pathways is still active after incubation with HDL. Furthermore, HDL inhibited the activation of c-Src kinase within 30 seconds of Ox-PAPC treatment (Figure 3A), a time insufficient for Ox-PAPC degradation. Thus, our data suggest that degradation of Ox-PAPC is not the mechanism by which HDL inhibits Ox-PAPC signaling. Rather, we propose that HDL acts in a rapid and sustained manner to differentially regulate certain Ox-PAPC-induced signaling pathways.

One potential mechanism mediating the protective effects of HDL on Ox-PAPC signaling is caveolar stabilization. Caveolae are small cholesterol-rich invaginations of the plasma membrane that maintain numerous signaling mole-

Figure 3. HDL alters the activation of several signal transduction pathways induced by Ox-PAPC. HAECs were either untreated (control) or treated with Ox-PAPC (50 μg/mL) for various time points in the presence and absence of human HDL (100 μg/mL). Protein levels of (A) c-Src kinase Y418 (top panel), normalized to total c-Src kinase (middle panel), and (B) STAT3 Y705 (top panel), normalized to total STAT3 (middle panel), were analyzed at 5 minutes after Ox-PAPC treatment using Western analysis. C, SREBP activation (top panel) at 2 hours after Ox-PAPC treatment was also measured by Western and normalized to GAPDH (bottom panel). Protein levels of (D) eNOS S1177 (top panel) and (E) eNOS T (top panel) at 1 hour after Ox-PAPC treatment were also analyzed; both were normalized to total eNOS levels (C and D, middle panel). F, Superoxide production, as measured using the NBT assay, was analyzed in HAECs treated with media alone or Ox-PAPC (50 μg/mL) for 1 hour. A to E, Densitometry was performed and fold activation is represented graphically (bottom panel). All experiments were repeated at least 3 times and representative blots are shown. F, Individual treatments were performed in 6 wells and data represent mean±SD of 3 experiments and *P<0.05 indicates significance (comparison between Ox+HDL and Ox groups).
cules in their inactive states.29 Previously, we reported that treatment of HAECs with Ox-PAPC depleted cholesterol from the plasma membrane and disrupted caveolae.8 Pretreatment of HAEC with cholesterol for 2 hours (presented in a complex with cyclodextrin) reversed caveolar disruption and partially inhibited Ox-PAPC–induced IL-8 and LDL receptor expression. In the current studies, we have demonstrated that HDL inhibits Ox-PAPC–induced activation of c-Src (Figure 3A) and STAT3 (Figure 3B), which are present in caveolae29,30 and inhibited IL-8 transcription by Ox-PAPC.11 HDL also inhibited Ox-PAPC–induced activation of SREBP (Figure 3C), which is likely mediated by the depletion of caveolar cholesterol induced by Ox-PAPC.8 The ability of HDL to prevent Ox-PAPC-mediated cholesterol depletion likely plays an important role in inhibiting the induction of chemokines and SREBP target genes by Ox-PAPC. However, the cholesterol donating properties of HDL do not fully explain its protective potential. Cholesterol pretreatment did not inhibit IL-8 and MCP-1 induction by Ox-PAPC12 to the same extent as HDL. Also, cholesterol pretreatment, unlike HDL treatment, does not regulate c-Src kinase activation (data not shown) or the induction of UPR genes by Ox-PAPC.12 Furthermore LDL, which can also reverse cholesterol depletion by Ox-PAPC, did not inhibit the UPR response and only modestly inhibited chemokine induction (data not shown). Therefore, HDL has additional properties, beyond donating cholesterol, which contribute to its anti-inflammatory profile. These studies also demonstrate that HDL is more effective than apoB-containing lipoproteins (ie, LDL) at inhibiting the induction of proinflammatory and UPR genes by Ox-PAPC.

Our data suggest another potential mechanism by which HDL inhibits the inflammatory, sterol regulatory, and UPR pathways induced by Ox-PAPC, that is, through an HDL-regulated decrease in Ox-PAPC-induced superoxide formation (Figure 3F). Superoxide has been implicated in the increased synthesis of inflammatory molecules, sterol regulatory pathways, and UPR.31–33 The decrease in superoxide production may be attributable to changes in the activity of NADPH oxidase, a decrease in eNOS uncoupling, or changes in other pathways mediating superoxide production. Our previous studies demonstrated the uncoupling of eNOS by Ox-PAPC, mediated through an increase in S1177 phosphorylation, and a decrease in T495 phosphorylation.9 In this study, we demonstrate that pretreatment with HDL does not affect S1177 phosphorylation but reverses the T495 dephosphorylation (Figure 3D, 3E). Dephosphorylation of eNOS at T495 has been associated with uncoupling of eNOS,34 in which case our data would suggest that HDL reverses Ox-PAPC–induced eNOS uncoupling. Other groups have associated dephosphorylation of T495 with increased activity of eNOS,35 in which case our data would suggest that HDL increases Ox-PAPC–induced eNOS activity, which might (in the context of increased superoxide produced by Ox-PAPC and eNOS in its coupled state) generate NO and decrease oxidative stress. The ability of HDL and its mimetics to prevent eNOS uncoupling has been reported by a number of investigators.36,37 Although the precise mechanism regulating

Figure 4. HDL does not inhibit the induction of redox genes by oxidized phospholipids. HAECs were either untreated (control) or treated for 4 hours with Ox-PAPC (50 μg/mL) in the presence and absence of human HDL (100 μg/mL). A, HO-1 (left panel) and heat-shock protein A1A (right panel) mRNA levels were analyzed using quantitative real-time polymerase chain reaction. B, HO1 protein expression (top panel), normalized to GAPDH (bottom panel), was analyzed using Western analysis. C, HO-1 mRNA levels in untreated HAECs (control) or HAECs treated for 4 hours with PEIPC (1 μg/mL) in the presence and absence of human HDL (100 μg/mL), were analyzed using quantitative real-time polymerase chain reaction. A and C, Individual treatments were performed in triplicate and data represent mean±SD and *P<0.05 indicates significance (comparison between Ox+HDL and Ox groups). B, Representative Western blot is shown. All experiments were repeated at least 3 times.
superoxide production by Ox-PAPC has yet to be determined, our data suggest that HDL pretreatment decreases the generation of superoxide by Ox-PAPC, thus contributing to the anti-oxidant and anti-inflammatory profile of HDL.

In contrast to the anti-inflammatory effect of HDL on Ox-PAPC signaling, HDL does not inhibit and may actually enhance the antioxidant effects of Ox-PAPC and PEIPC. HO-1 is an important antioxidant molecule, and the mRNA levels of HO-1 are strongly increased by Ox-PAPC in HAECS. Pretreatment with 100 μg/mL HDL (and even concentrations up to 400 μg/mL for 24 hours, data not shown) did not inhibit the induction of HO-1 expression by Ox-PAPC (Figure 4A). In fact, HO-1 expression was slightly higher in Ox-PAPC–treated cells pre-incubated with HDL. Recently, Kruger et al.38 reported increased levels of HO-1 in Sprague-Dawley rats fed with the apoA-1 mimic peptide, D-4F. Transcriptional regulation of HO-1 is complex and can be mediated by a number of signal transduction pathways. The most commonly studied pathway regulating HO-1 expression in other systems is through the induction of oxidative stress and subsequent activation of Nrf2. Other pathways, however, such as activation by transcription factors including AP-1 and NF-kB have also been reported.25,26 We previously reported that Ox-PAPC treatment does not activate NF-kB or cause significant activation of AP-1. In the current studies, we have demonstrated that Ox-PAPC treatment increased the nuclear translocation of Nrf2 (Figure 5A). Increased nuclear Nrf2 likely reflects increased levels of active transcription factor. HDL, however, did not reduce that activation of Nrf2 mediated by Ox-PAPC (Figure 5B). Thus, we propose that HDL preserves the induction of HO-1 by Ox-PAPC by preserving activation of the pathway responsible for Nrf2 nuclear translocation, which is the major transcriptional regulator of HO-1.

Conclusions
In summary, the current studies have demonstrated that HDL has a protective role in regulating Ox-PAPC signaling by inhibiting the inflammatory pathways induced by Ox-PAPC while preserving and potentially upregulating the anti-inflammatory and antioxidant pathways induced by Ox-PAPC. Taken together, these findings suggest an additional mechanism by which HDL inhibits atherosclerosis: by directly altering the inflammatory properties of oxidized phospholipids that are present in atherosclerotic lesions and other sites of chronic inflammation to become anti-inflammatory and antioxidant.

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Disclosures
None.

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