All ApoB-Containing Lipoproteins Induce Monocyte Chemotaxis and Adhesion When Minimally Modified
Modulation of Lipoprotein Bioactivity by Platelet-Activating Factor Acetylhydrolase

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Abstract—Mildly oxidized LDL has many proinflammatory properties, including the stimulation of monocyte chemotaxis and adhesion, which are important in the development of atherosclerosis. Although ApoB-containing lipoproteins other than LDL enter the artery wall and undergo oxidation, very little is known regarding their proinflammatory potential. LDL, IDL, VLDL, postprandial remnant particles, and chylomicrons were mildly oxidized by fibroblasts overexpressing 15-lipoxygenase (15-LO) and tested for their ability to stimulate monocyte chemotaxis and adhesion to endothelial cells. When conditioned on 15-LO cells, LDL, IDL, but not VLDL increased monocyte chemotaxis and adhesion 4-fold. Chylomicrons and postprandial remnant particles were also bioactive. Although chylomicrons had a high 18:1/18:2 ratio, similar to that of VLDL, and should presumably be less susceptible to oxidation, they contained (in contrast to VLDL) essentially no platelet-activating factor acetylhydrolase (PAF-AH) activity. Because PAF-AH activity of lipoproteins may be reduced in vivo by oxidation or glycation, LDL, IDL, and VLDL were treated in vitro to reduce PAF-AH activity and then conditioned on 15-lipoxygenase cells. All 3 PAF-AH–depleted lipoproteins, including VLDL, exhibited increased stimulation of monocyte chemotaxis and adhesion. In a similar manner, lipoproteins from Japanese subjects with a deficiency of plasma PAF-AH activity were also markedly more bioactive, and stimulated monocyte adhesion nearly 2-fold compared with lipoproteins from Japanese control subjects with normal plasma PAF-AH. For each lipoprotein, bioactivity resided in the lipid fraction and monocyte adhesion could be blocked by PAF-receptor antagonists. These data suggest that the susceptibility of plasma lipoproteins to develop proinflammatory activity is in part related to their 18:1/18:2 ratio and PAF-AH activity, and that bioactive phospholipids similar to PAF are generated during oxidation of each lipoprotein. Moreover, LDL, IDL, postprandial remnant particles, and chylomicrons and PAF-AH–depleted VLDL all give rise to proinflammatory lipids when mildly oxidized. (Arterioscler Thromb Vasc Biol. 1999;19:1437-1446.)

Key Words: atherosclerosis • lipid peroxidation • platelet-activating factor acetylhydrolase • autoantibodies • LDL oxidation

Evidence from both in vitro and in vivo studies suggests that oxidation of LDL may contribute to early atherosclerotic lesion formation. The extent of LDL oxidation appears to influence which of a variety of potential atherogenic properties of oxidized LDLs predominate at a given point in time. When LDL is less oxidized, ie, minimally modified LDL (mm-LDL), it stimulates monocyte chemotaxis, adherence to and transmigration through endothelial cells, as well as expression of several growth factors such as macrophage colony-stimulating factor. These and other “bioactive” properties of mm-LDL very likely contribute to the development of atherosclerosis. Many of the proinflammatory properties of mm-LDL may result from the formation of oxidized phospholipids. Several of these oxidized phospholipids have been identified by liquid chromatography/mass spectrometry and at least 1 group of proinflammatory compounds results from oxidative degradation of arachidonic acid within the phospholipid. Studies by our laboratory have suggested that oxidation of other phospholipid polyunsaturated fatty acids (PUFAs), such as linoleic acid, may also contribute to the generation of bioactive phospholipids.

Most investigations of lipoprotein oxidation in relation to atherosclerosis have focused on LDL, which has been isolated by ultracentrifugation over the broad density range of...
1.019 to 1.063 g/mL. More recently it has become apparent that small dense subfractions of LDL have enhanced susceptibility to copper-mediated oxidation and may have greater atherogenic potential. However, the relevance of in vitro copper-mediated oxidation has been questioned and differences in the susceptibility of LDL subfractions to become “minimally modified” and bioactive have not been studied. There is also increasing evidence that more triglyceride-rich lipoproteins (IDLs, VLDLs, and postprandial remnant particles) may also enter the artery wall and contribute to the lipid accumulation in lesions.11-12 Several of these particles have been previously shown to be susceptible to oxidation in vitro.13-15 Because lipid-rich lipoproteins such as IDLs, VLDLs, and chylomicrons as well as their remnant particles may be an important source of PUFA-containing phospholipids, it is conceivable that their oxidation may generate substantial bioactivity. In addition, studies indicate that in humans oxidized lipids in the diet are absorbed by the small intestine and are transported in chylomicrons to the circulation, where they can be incorporated into VLDL by the liver, or perhaps directly transferred to other lipoproteins.16,17 Thus, postprandial lipoproteins may already contain oxidized lipids that could be readily converted to proinflammatory lipids after exposure to mild oxidative stress. The current studies evaluate the susceptibility of various lipoproteins and lipoprotein subfractions to generate proinflammatory particles when exposed to mild oxidative stress, as measured by their ability to stimulate monocyte chemotaxis and monocyte adherence to endothelial cells.

Methods

Lipoprotein Isolation

VLDL (d=1.006 g/mL), IDL (d=1.006 to 1.019 g/mL), LDL (d=1.022 to 1.063 g/mL), as well as dense LDL (1.040 to 1.063 g/mL) and buoyant LDL (1.026 to 1.032 g/mL) were isolated by density gradient ultracentrifugation from pooled human plasma containing a final concentration of gentamicin 0.22 mmol/L, chloramphenicol 0.15 mmol/L, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone 1 mmol/L, benzamidine 2 mmol/L, and EDTA 300 mmol/L. After isolation, the lipoproteins were dialyzed in PBS containing EDTA for 24 hours, stored at 4°C in the dark, and used within 1 week. EDTA was removed from the LDL just before each oxidation experiment by dialysis for 20 hours at 4°C in the dark with 2 6-L changes of PBS. For some experiments, we isolated LDL and VLDL from pooled plasma from several “normal” Japanese subjects as well as from Japanese subjects with a deficiency in plasma platelet-activating factor acetylhydrolase (PAF-AH) (generously provided by Drs Subbaiah and Miwa). The affected individuals have a recently characterized missense mutation near the active site of the PAF-AH.20 Chylomicrons were isolated by overlayering 6 mL of plasma with 6 mL of saline and ultracentrifuging the mixture for 20 minutes in a SW 41 rotor at 35 000 rpm. The chylomicrons were washed by repeating the above process 2 additional times. A postprandial “remnant”-enriched lipoprotein fraction (1.006<d<1.021 g/mL) was also prepared by density gradient ultracentrifugation. For chylomicron and remnant particle isolation, blood was collected from volunteers 4 hours after a standard high-fat meal. All lipoprotein fractions were checked for contamination by albumin or other lipoproteins by acrylamide gel analysis. Chylomicron preparations contained predominantly ApoB48-containing particles and postprandial remnant preparations contained primarily ApoB100-containing particles and some ApoB48-containing particles. Lipopolysaccharide content of lipoproteins was measured by the gel clot method (Sigma Chemical) and the ApoB100 content of lipoproteins was determined by using immunoprecipitation analysis (Inctar Corp).

Lipoprotein Oxidation

The formation of conjugated dienes was measured as previously described,21 by incubating lipoproteins at 150 μg ApoB100/mL with 5 mmol/L copper sulfate in 1 mL of PBS at 30°C. The absorbance at 234 nm was measured continuously in a Uvikon 951 spectrophotometer. For presentation of conjugated diene data, the first derivative of the rapid phase of oxidation was calculated and its intercept with the x axis (lag time) determined. In some experiments, the extent of copper-mediated lipid oxidation was determined by the generation of malondialdehyde products (thiobarbituric acid–reactive substances [TBARS] assay) by the method of Yagi22 with fluorescence intensity measured at 553 nm and excitation at 515 nm.

Fatty Acid Determinations

Lipids from lipoprotein samples were extracted by a modification of the method of Folch et al.23 The fatty acids were transmethylated and analyzed in a Varian gas chromatograph Model 3700, equipped with a column of 10% Silar 5CP on a Gas Chrom QII, 100/120 mesh as described.24

Vitamin E Content

α-Tocopherol was measured by HPLC as described previously.18 α-Tocopherol acetate was prepared in 100% ethanol and used as an extraction internal standard and for standard curve preparation. Actual concentrations of α-tocopherol were determined by measuring absorbance of prepared solutions and calculating concentrations based on known spectral data. Calculations were determined from a standard curve of peak area ratios of sample/internal standard.

Cell Culture Procedures

Murine fibroblasts expressing high levels of intracellular 15-lipoxygenase (15-LO) were established by infection with a retroviral vector as previously described.25,26 We previously demonstrated that incubation of LDL on these 15-LO cells, but not on control fibroblasts expressing β-galactosidase, generates a modified LDL that meets all the criteria for mm-LDL.4,15 and in particular can stimulate monocyte chemotaxis and adhesion to endothelial cells.27,28 Although there is controversy regarding the mechanisms of in vivo modification of lipoproteins, 15-LO cells are used in this system not to illustrate the importance of 15-LO in lipoprotein oxidation, but as a reliable method of generating mm-LDL, as we previously demonstrated. Cells were grown in Dulbecco’s modified essential medium with high glucose (10 mmol/L), containing 10% FCS and G418 sulfate (50 mg/mL) at 37°C and in 5% CO2. Fibroblasts were plated on 96- well plates at 35 000 cells per well and grown for 2 days until nearly confluent. The cells were washed free of serum, and lipoproteins (LDL, IDL, and VLDL) at 30 to 150 μg ApoB100/mL were then incubated with the fibroblasts at 37°C for 20 hours in Ham’s F-10 media. Chylomicrons and postprandial remnant particle fractions were incubated on 15-LO cells based on equal triglyceride concentrations. TBARS were determined to assess the extent of lipoprotein oxidation and bioactivity was assessed as described below.

In additional experiments, lipoprotein fractions were modified on endothelial cell/smooth muscle cell cocultures as described by Navab et al.4,5 Human aortic smooth muscle cells were initially plated on 96-well plates at 35 000 cells per well and grown for 2 days until nearly confluent. Then, human umbilical vein or porcine aortic endothelial cells were plated on the smooth muscle cells and grown until confluent. Lipoproteins were then incubated on the coculture at 37°C for 20 hours in RPMI media in the presence of 5% lipoprotein-deficient serum and bioactivity of the supernatant was then assessed.

Human monocytes were isolated from blood collected in 4 mmol/L EDTA. A monocyte-enriched fraction was isolated by density ultracentrifugation at 22°C, using Histopaque 1077 (Sigma Chemical). The cells were then plated in RPMI 1640 (BioWhitaker)+10% homologous serum for 3 hours at 37°C. Nonadherent cells were washed off and the adherent monocytes were released, using PBS containing 0.18% EDTA, and were then washed twice in
TABLE 1. Composition of Lipoproteins

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>CHOL (mg)/ApoB (mg)</th>
<th>Trig (mg)/ApoB (mg)</th>
<th>VIT E (µg)/ApoB (mg)</th>
<th>VIT E (µg)/PUFA (mg)</th>
<th>PAF-AH Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>1.5±0.10</td>
<td>0.4±0.55</td>
<td>7.1±1.67</td>
<td>14.6±4.6</td>
<td>1636.9±311.7</td>
</tr>
<tr>
<td>IDL</td>
<td>1.5±0.30</td>
<td>1.4±0.25</td>
<td>11.9±2.95</td>
<td>18.2±6.7</td>
<td>1407.4±586.1</td>
</tr>
<tr>
<td>VLDL</td>
<td>2.4±0.83</td>
<td>11.8±6.18</td>
<td>35.4±11.28</td>
<td>14.9±6.3</td>
<td>1933.4±579.7</td>
</tr>
</tbody>
</table>

Data are mean±SD values of 6 or more different lipoprotein preparations. CHOL indicates cholesterol; Trig, triglycerides; and VIT E, vitamin E.

* Nanomoles of PAF degraded per hour per milligram of ApoB.

Monocyte Chemotaxis Assay

Assays were performed in a chemotaxis chamber (Neuro Probe Inc) with a polycarbonate filter (Poretics) of 5-µm pore size separating the upper and lower wells. The lower wells were filled with 29 µL of supernatant (diluted 1:5 in 0.1% BSA/Tyrode’s salt solution) from either fibroblasts or endothelial monolayers incubated overnight. The chambers were treated as previously described by Navab et al. The monocytes that migrated from the upper chamber to the lower surface of the filter were then counted by using a light microscope and expressed as cells per high-power field. The results of at least 4 to 8 wells were averaged for each experimental condition.

Monocyte Adhesion Assay

The assay, with minimal modifications, was performed as described by Navab et al. Lipoproteins conditioned in media alone, by fibroblasts in F-10 media, or by endothelial/smooth muscle cell cocultures in RPMI for 20 hours were transferred to confluent porcine aortic endothelial monolayers in 96-well tissue culture plates and the plates were incubated for 4 hours at 37°C. The supernatants were then removed and the endothelial monolayers washed twice with RPMI 1640. THP-1 cells (a monocyte-like cell line) were placed on the endothelial cells at 45 000 cells per well, and the plates were incubated for 20 minutes at 37°C. The suspension was removed, and the cells were washed vigorously (at least 3 times) to remove all but the firmly adherent THP-1 cells. The number of adherent THP-1 cells was determined in 4 high-power fields per well and the results of 4 to 8 separate wells were averaged for each experiment. In some experiments, we separated conditioned LDL from the aqueous supernatant by passing the incubation mixture through a membrane cone with a molecular weight cutoff of 25 000 (Amicon). Both the retained lipoproteins (resuspended in Tyrode’s salt solution) and ultrafiltrate were then tested individually for chemotaxis activity. In additional studies, we extracted the total lipids with chloroform/methanol by the addition of alkaline phosphate substrate.

Chemiluminescent Immunoassay for Antibody Binding

Supernatants containing conditioned lipoproteins or native lipoprotein samples were diluted to 10 µg/mL in PBS buffer (containing 0.27 mmol/L EDTA) and were plated onto 96-well white round-bottomed high binding Microfluor (Dynex Technologies, Inc) microtiter plates overnight at 4°C. The wells were washed 4 times with PBS buffer and blocked with PBS buffer containing 1% BSA for 30 minutes. Natural monoclonal autoantibodies (E0 autoantibodies) directed against epitopes of oxidized LDL were cloned from ApoE-deficient mice as previously described. E0 autoantibodies were incubated with the plated lipoproteins for 1 hour at room temperature. The amount of antibody bound was measured with alkaline phosphatase–labeled goat anti-mouse-IgM (Sigma) (in TBS buffer containing 1% BSA), using a chemiluminescent technique previously described. Data are expressed in relative chemiluminescent light units.

Additional Assays

In some experiments, lipoproteins were incubated with serine esterase inhibitors aminobenzylsulfonyl fluoride (ABSF) (Sigma) at 25 to 400 µmol/L or phenylmethysulfonyl fluoride (PMSF) (Sigma) at 3 mmol/L for 60 to 90 minutes to irreversibly inhibit PAF-AH. Lipoprotein samples were then dialyzed free of excess inhibitors and EDTA and then conditioned on 15-LO cells as described above.

PAF-AH activity in lipoproteins was measured by the method of Stafforini et al. In brief, diluted tritiated PAF (Du Pont–NEN) was incubated with 4 µg of protein from each lipoprotein fraction for 30 minutes before the reaction was stopped by the addition of a 50-µL mixture of acetic acid and sodium acetate. The cleaved tritiated acetate product was separated from the intact PAF substrate by reverse-phase octadecyl silica gel column chromatography. The PAF-AH activity is expressed as nanomoles of PAF hydrolyzed per hour per milligram of protein or per milligram of ApoB100.

The presence of PAF-AH protein in various lipoprotein classes was demonstrated by western blot analysis. After lipid extraction, ~10 µg of protein was resuspended in Tris-glycine reducing buffer, heated at 70°C for 10 minutes, and run on 4% to 12% Tris-glycine gradient gels (Novex) in 25 mmol/L Trizma base, 192 mmol/L glycine, and 1% SDS for 2 hours at 125 V. Samples were transferred to nitrocellulose paper in Novex transfer units over 16 hours at 4°C at 50 to 100 V in a transfer buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol. Nonspecific binding sites were blocked with Superblock (Pierce) and 0.1% human serum albumin and incubated overnight with a 1:500 dilution of rabbit anti-human LDL PAF-AH (a generous gift of Dr Colin Macphee). After extensive washing, an alkaline phosphatase–conjugated goat anti-rabbit IgG (diluted 1:10 000) was added and the signal detected by the addition of alkaline phosphate substrate.

Figure 1. Western blot of PAF-AH in LDL, IDL, and VLDL. After lipid extraction, ~10 µg of protein from each lipoprotein was subjected to SDS–gel electrophoresis under reducing conditions and transferred to nitrocellulose. Immunodetection of PAF-AH was performed with a 1:500 dilution of rabbit anti-human LDL PAF-AH (B). PAF-AH standard was prepared from LDL, as described by Tew et al, and demonstrated several bands with molecular masses of ~57 kDa. Columns 1 through 9 represent molecular mass standards, HDL, LDL, IDL, total VLDL, VLDLlight (d=1.006 g/mL), VLDLmedium (1.006<d<1.068 g/mL), and pure VLDL.
Results

Composition of Lipoprotein Particles

The compositions of isolated LDL, IDL, and VLDL particles are shown in Table 1. Data are shown normalized to mg of ApoB100 unless stated otherwise so that comparisons between lipoprotein classes may be made per lipoprotein particle. Although the vitamin E content per particle was significantly greater in VLDL, the vitamin E content per PUFA was not different between each lipoprotein. Although PAF-AH activity when expressed per total protein was greatest in LDL, the level of enzyme activity per ApoB100 protein, ie, per particle, was similar in LDL, IDL, and VLDL. The presence of PAF-AH protein in each ApoB-containing lipoprotein was confirmed by western blot analysis, using a polyclonal antibody to PAF-AH (Figure 1). The PAF-AH protein band in HDL was only faintly visible, consistent with the 50–100-fold lower specific activity of PAF-AH measured in this lipoprotein. In contrast, the fatty acid composition between lipoprotein classes may be made per lipoprotein particle. Although the vitamin E content per particle was significantly greater in VLDL, the vitamin E content per particle was more readily oxidized when exposed to copper and had significantly reduced lag times compared with buoyant LDL (167.6±99.3 versus 252.1±70.8 minutes). As shown in Figure 2B, there was also a similar order of susceptibility to copper-mediated oxidation among the different lipoprotein fractions as measured by the formation of TBARS. LDL was first to undergo oxidation, followed by IDL and then VLDL. Similar patterns of oxidation were seen when oxidation was mediated by 2,2-azobis (2-amidinopropane) dihydrochloride rather than by copper (data not shown). As previously reported,10,18 dense LDL particles were more readily oxidized when exposed to copper and had significantly reduced lag times compared with buoyant LDL (167.6±99.3 versus 252.1±70.8 minutes).

Bioactivity Generated From Lipoproteins Conditioned on 15-LO Cells

We previously showed that incubation of LDL with 15-LO–overexpressing fibroblasts mildly oxidizes LDL25,26 and converts it to a mm-LDL that induced greater monocyte chemotaxis and adhesion.27 To evaluate whether mild oxidative stress would have similar effects on other lipoproteins, we conditioned LDL, IDL, and VLDL on 15-LO cells and then compared the supernatants from these incubations for their chemotactic activity and induction of monocyte adhesion to endothelial cells. Figure 3A shows that conditioning LDL and IDL on 15-LO cells significantly increased their ability to stimulate monocyte chemotaxis. In contrast, conditioning VLDL on 15-LO cells only modestly (and not significantly) increased its stimulation of monocyte chemotaxis. In several

Susceptibility of Lipoproteins to Copper-Mediated Oxidation

Susceptibility of LDL, IDL, and VLDL to copper-mediated oxidation was measured by several different methods. Conjugated diene formation revealed that based on equal amounts of ApoB100, ie, equal numbers of particles, LDL consistently oxidized more rapidly than did the other 2 lipoproteins (Figure 2A). The lag time for VLDL oxidation was significantly longer than that of other lipoproteins. The peak level of conjugated diene formation was higher for both IDL and VLDL compared with LDL, consistent with their greater lipid content. As shown in Figure 2B, there was also a similar order of susceptibility to copper-mediated oxidation among the different lipoprotein fractions as measured by the formation of TBARS. LDL was first to undergo oxidation, followed by IDL and then VLDL. Similar patterns of oxidation were seen when oxidation was mediated by 2,2-azobis (2-amidinopropane) dihydrochloride rather than by copper (data not shown). As previously reported,10,18 dense LDL particles were more readily oxidized when exposed to copper and had significantly reduced lag times compared with buoyant LDL (167.6±99.3 versus 252.1±70.8 minutes). As shown in Figure 2B, there was also a similar order of susceptibility to copper-mediated oxidation among the different lipoprotein fractions as measured by the formation of TBARS. LDL was first to undergo oxidation, followed by IDL and then VLDL. Similar patterns of oxidation were seen when oxidation was mediated by 2,2-azobis (2-amidinopropane) dihydrochloride rather than by copper (data not shown). As previously reported,10,18 dense LDL particles were more readily oxidized when exposed to copper and had significantly reduced lag times compared with buoyant LDL (167.6±99.3 versus 252.1±70.8 minutes).
additional experiments, HDL was also conditioned on 15-LO cells, and like VLDL, demonstrated little or no bioactivity compared with lipoproteins incubated in media alone. Incubation of LDL and IDL conditioned on 15-LO cells with endothelial cells markedly increased the subsequent adherence of THP-1 cells to endothelial cells, whereas conditioned VLDL did not do so. Lipoplysaccharide was measured in each lipoprotein class and was consistently below our detection level of 0.5 ng/mL, a value that does not stimulate monocyte chemotaxis or adhesion in our assays.

Recent investigations have highlighted the increased risk for cardiovascular disease associated with small dense LDL. Several mechanisms for this relation have been proposed, including enhanced susceptibility to oxidation of small dense LDL. We therefore also undertook studies to determine whether small dense LDL subfractions were more readily converted to a bioactive mm-LDL than were larger buoyant LDL. As shown in Figure 4, after conditioning on 15-LO cells, dense LDL stimulated greater chemotaxis and monocyte adhesion than did buoyant LDL.

Relation Between Lipoprotein Oxidation and Bioactivity

The large differences in bioactivity of LDL and IDL compared with VLDL after incubation on 15-LO cells could not readily be explained by differences in oxidation, at least as measured by nonspecific measures of lipid peroxidation, such as TBARS. Although 15-LO–conditioned IDL was as bioactive as LDL, its level of TBARS/ApoB100 after conditioning on 15-LO cells was significantly lower than that of LDL and comparable with that of VLDL (LDL, 34 ± 10.7 nmol/mg ApoB; IDL, 10.7 ± 8.7 nmol/mg ApoB; and VLDL, 15.4 ± 10.7 nmol/mg ApoB). This is consistent with several previous reports where measures of TBARS have not corresponded to the development of LDL bioactivity after incubation with 15-LO–overexpressing cells. One interpretation of these data is that oxidation is not directly related to the development of bioactivity. However, inhibition of oxidation by the addition of EDTA to the incubation mixture or by using probucol-enriched lipoproteins consistently reduced generation of lipoprotein-induced bioactivity (data not shown). It is known that reducing the concentration of LDL exposed to a given concentration of copper will result in greater oxidation. Therefore, we substantially reduced the concentration of LDL incubated with the 15-LO cells from 200 hours to 20 hours and the supernatant from these incubations was used for stimulation of monocyte chemotaxis as described in the legend to Figure 3 and Methods. The results of at least 4 to 8 wells were averaged for each experimental condition. Shown are mean ± SE values from 4 experiments after subtracting out the NCC values. B, Porcine aortic endothelial cells were treated for 4 hours with buoyant LDL or dense LDL (at 150 μg ApoB100/mL in F-10) that had been incubated for 20 hours on 15-LO fibroblasts or in media alone. Adhesion assays were performed as described in Figure 3 and Methods. Data reflect mean ± SE values (after subtracting out the number of THP-1 cells that adhere when endothelial cells are pretreated with media alone) from 6 different experiments. *Significant at P < 0.01, compared with buoyant LDL.
other E0 antibodies that are directed toward LDL phospholipid oxidation epitopes were used (data not shown). The formation of oxidized phospholipid epitopes appeared to reflect the inherent susceptibility of these lipoproteins to copper-mediated oxidation, as well as their bioactivity. This is demonstrated in Figure 5, which shows the results of experiments in which LDL, at decreasing concentrations of ApoB100 and then assayed for E06 binding (A) and chemotaxis activity (B). Chemotaxis activity is expressed as relative chemotactic units (%) compared with that obtained for LDL incubated on 15-LO cells at 30 μg/mL. Chemotaxis data are normalized per equivalent ApoB100 amounts. RLU indicates relative light units. Shown are representative experiments.

**Substantial Bioactivity Resides in the Lipid Fractions of Lipoproteins Conditioned on 15-LO Cells**

Lipid extracts of cell-conditioned LDL and IDL were prepared and when tested for stimulation of chemotaxis, this fraction contained >70% of the original bioactivity. When experimental conditions were modified to enhance cell-mediated oxidation of VLDL (as described above), this lipid extract was also bioactive (data not shown). In a similar manner, separating lipoproteins after their incubations on 15-LO cells from the rest of the supernatant by ultrafiltration through a membrane with a mw cutoff of 25 000 revealed that the bioactivity was predominantly associated with the lipoprotein particles and not the aqueous supernatant (data not shown). We and other investigators have recently shown that generation of bioactivity appears, in part, to result from the oxidative degradation of lipoprotein phospholipids. At least a portion of these bioactive substances resemble PAF in structure, and appear to activate cell adhesion through the PAF receptor. To determine whether the bioactivity of each 15-LO–conditioned lipoprotein was related to the generation of PAF-like particles, we added structurally different PAF-receptor antagonists to the endothelial cells before and during their incubation with the lipoproteins. As shown in Figure 6, the PAF-receptor antagonist Lau 203 completely blocked the ability of conditioned LDL and IDL to stimulate monocyte adherence. In a similar manner, it appears that even the modest bioactivity of conditioned VLDL was also inhibited by the PAF-receptor antagonist BNS52021. The vehicle for the PAF-receptor antagonists had no effect on monocyte adherence nor was the PAF-receptor antagonist Lau 203 able to reduce the stimulation of monocyte adhesion induced by addition of tumor necrosis factor or lipopolysaccharide (data not shown).

**Effect of PAF-AH on Bioactivity of Lipoproteins Conditioned on 15-LO Cells**

Lipoproteins contain several different enzymes that may have the ability to degrade bioactive oxidized phospholipids. One...
such enzyme that is particularly prominent in ApoB100-containing lipoproteins is PAF-AH. It has been reported that the majority of PAF-AH in plasma resides in LDL.38,39 As shown in Figure 1, we demonstrated the presence of PAF-AH protein in IDL and VLDL in addition to LDL. To compare the role of PAF-AH activity in degrading bioactive phospholipids in different ApoB100-containing lipoproteins, we pretreated LDL, IDL, and VLDL with serine esterase inhibitors, which irreversibly inhibit PAF-AH, conditioned them on 15-LO cells, and then repeated the assays of bioactivity. Each lipoprotein fraction treated with PMSF or ABSF had lipoprotein-associated PAF-AH activity that was reduced to <20% of their basal activity. In comparison, lipoproteins conditioned on 15-LO cells overnight only lose 10% to 15% of their PAF-AH activity. All of the PAF-AH–depleted lipoproteins, including VLDL, showed enhanced bioactivity compared with the PAF-AH–replete lipoproteins (Figure 7).

In separate experiments, E06 antibody binding to PAF-AH–depleted lipoproteins after their modification by 15-LO cells was increased to a greater extent than was binding to 15-LO–conditioned PAF-AH–replete lipoproteins (data not shown). These data support that lipoproteins depleted of PAF-AH generate greater amounts of bioactive oxidized phospholipids when mildly oxidized. Reducing PAF-AH activity in native lipoproteins or those conditioned in media alone did not enhance their bioactivity (data not shown).

Enzyme inhibitors may have other unknown effects on cells or lipoproteins. Therefore, we compared LDL isolated from plasma from Japanese subjects who have been identified as having an “inactivating” mutation in the gene for PAF-AH to LDL from Japanese control subjects.20,40 The lipid (cholesterol, triglyceride, and phospholipid) and fatty acid composition of the lipoproteins as well as the plasma vitamin E levels were not different in the 2 groups (data not shown). The LDL from individuals homozygous for this mutation contained no PAF-AH activity, whereas activity was present in LDL from the control subjects (720 nmol PAF/h/mg of protein). When conditioned on 15-LO cells, LDL from plasma PAF-AH–deficient subjects stimulated greater monocyte adhesion than did LDL samples from the control subjects (Figure 8). Treatment of Japanese control LDL with ABSF completely inactivated PAF-AH, and after conditioning on 15-LO cells these LDL samples demonstrated bioactivity equal to PAF-AH–deficient samples. A similar increase in bioactivity resulted when VLDL, isolated from these same PAF-AH–deficient patients, was conditioned on 15-LO cells (data not shown). These data illustrate the potentially important antinflammatory role of PAF-AH in all ApoB100-containing lipoproteins.

Bioactivity of Postprandial Lipoproteins
Conditioned on 15-LO Cells and Smooth Muscle/Endothelial Cell Cocultures

Recent studies have suggested postprandial hyperlipoproteinemias, particularly elevations in remnant particles, may contribute to lesion formation.41–43 We therefore evaluated chylomicrons and a remnant-enriched fraction that were isolated ≈4 hours after a high-fat meal. As shown in Table 2, postprandial chylomicrons were markedly enriched in 18:1 and relatively low in 18:2 and 20:4. However, the vitamin E/PUFA ratio was very low in this lipoprotein fraction (3.9±1.9) compared with all other lipoproteins, and PAF-AH activity was essentially absent. In contrast, the postprandial remnant particles, predominantly comprised of hepatic derived lipoproteins, were less enriched with 18:1 but contained...
per-particle basis, mm-IDL and mm-LDL are relatively equal in their ability to stimulate monocyte chemotaxis and adhesion. With exposure to greater oxidative stress, VLDL can also acquire proinflammatory activity. Exposure of postprandial lipoproteins to mild oxidative stress also induces their transformation to bioactive particles. Although these experiments only demonstrated that minimally oxidized IDL, VLDL, and postprandial chylomicrons and remnant particles can increase monocyte chemotaxis and adhesion, it is likely that they will also have other proinflammatory properties associated with mm-LDL.4,44

Numerous studies have demonstrated that individuals at increased risk for atherosclerosis, such as individuals with diabetes, familial combined hyperlipidemia, or type III hyperlipidemia, have increased levels of non-LDL ApoB-containing lipoproteins.15,41,45,46 As a result, it has been suggested that remnant lipoproteins, in particular, may be proatherogenic, and in vitro studies have suggested several possible mechanisms by which particles such as IDL or PAF-AH depleted-VLDL may accelerate lesion formation.13 Moreover, animal studies have demonstrated that elevations in remnant particles can induce atherosclerosis.4,43 This has also been supported by studies of atherosclerosis progression in humans.42,48–50 Finally, several recent studies have demonstrated the presence of non-LDL lipids and lipoproteins in lesions in the artery wall,11,12 consistent with the concept that lipoproteins larger than LDL, such as IDL and VLDL, may contribute to lesion formation.

Comparisons between lipoprotein fractions revealed several features in common. Measurements of bioactivity generally reflected the susceptibility of these lipoproteins to copper-mediated oxidation. For example, compared with IDL and LDL, VLDL was more resistant to transition metal–mediated oxidation and less readily developed bioactivity. When conditions were changed to promote greater oxidation of VLDL, it too developed bioactivity. This provides some support for the relevance of in vitro measurements of copper-mediated lipoprotein oxidation.

Several lines of evidence suggest that at least a major portion of mm-lipoprotein bioactivity results from the generation of oxidized phospholipids, and this seemed true for each class of lipoproteins. In all lipoproteins tested, the majority of the bioactivity resided in the lipid fraction. In addition, during conditioning on 15-LO cells, all ApoB100-containing lipoproteins developed oxidation epitopes that were detected by endogenously produced murine autoantibodies that have been demonstrated to specifically bind oxidized phospholipids.8,32 By using this extremely sensitive and specific immunological assay of “oxidation-specific” epitopes, we were able to document the parallel relation between mild phospholipid oxidation and bioactivity. In addition, as described below, decreases in lipoprotein-associated PAF-AH, an enzyme that appears to degrade biologically active products of phospholipid oxidation, enhances lipoprotein proinflammatory activity. It is noteworthy that the stimulation of monocyte adhesion by each class of lipoprotein could be blocked by PAF-receptor antagonists. These data suggest that the source of bioactivity of each class of lipoprotein is in part related to the development of PAF or PAF-like lipid products. This is consistent with the findings of previous investigators who have demonstrated that bioactivity of mm-LDL appears

Evidence now supports the concept that the development of atherosclerosis is in many ways an inflammatory process that is initiated or exacerbated by hyperlipidemia. An early step in this process is the recruitment and adhesion of monocytes to sites of inflammation. Our findings demonstrate that all ApoB100-containing lipoproteins are susceptible to mild oxidation and acquire proinflammatory properties. These results were not dependent on the cell system used to induce mild oxidation, as similar bioactivity was generated when lipoproteins were incubated on smooth muscle/endothelial cell cocultures (data not shown). When compared on a

Figure 9. Stimulation of monocyte chemotaxis and adhesion by chylomicrons and postprandial remnant fractions. Chylomicrons (CHYO) (A) or postprandial remnant particles (PPRP) (B) were incubated at 75 μg of triglyceride/mL with 15-LO cells or endothelial/smooth muscle cell cocultures (CM) for 20 hours and the supernatant from these incubations tested for stimulation of monocyte chemotaxis as described in the legend to Figure 3 and Methods. The results of at least 4 wells were averaged for each experimental condition. Shown are mean±SE values from 4 experiments after subtracting out the values obtained for native lipoproteins.
related to specific products of oxidized phospholipids.\textsuperscript{3,8} Although our findings are consistent with the concept that PAF or PAF-like lipids increased monocyte chemotaxis through their interaction with the monocyte PAF receptor, our data do not exclude other alternatives.

The susceptibility of ApoB100-containing lipoproteins to undergo oxidation and develop bioactivity emphasizes the potentially important role of PAF-AH in these fractions. In each lipoprotein class, the loss of PAF-AH seemed to increase the level of bioactivity that developed when conditioned on cells. This was perhaps most evident in VLDL, where the decrease of PAF-AH activity by serine esterate inhibitors facilitated its transformation from a mild to a relatively potent inflammatory particle. However, such inhibitors may have nonspecific effects on cells and/or lipoproteins. Indeed, there is evidence that such inhibition of PAF-AH in some studies may reduce bioactivity of oxidized LDL.\textsuperscript{34}

To address these possibilities, we evaluated lipoproteins obtained from Japanese subjects with known mutations in their gene for the plasma form of PAF-AH. These individuals have plasma and lipoproteins that contain no measurable PAF-AH activity. Minimally oxidized LDL containing the inactive PAF-AH consistently induced greater monocyte adhesion. Similar results occurred with VLDL samples isolated from these PAF-AH–deficient subjects. These data support the concept that PAF-AH degrades bioactive oxidized phospholipids within oxidized lipoproteins. However, the exact role and in vivo relevance of plasma PAF-AH awaits studies of PAF-AH depletion and overexpression in animals as well as careful, long-term follow-up of patients with deficiencies of plasma PAF-AH.

Although there were many similarities between lipoprotein fractions in their response to mild oxidative stress, there were also some unique differences. For example, VLDL was in general less easily transformed to a bioactive particle. This may in part have resulted from its increased content of 18:1. We previously demonstrated that lipoproteins\textsuperscript{24,28,51} and liposomes\textsuperscript{9} enriched in 18:1 were less easily oxidized and less readily generated chemotactic activity.\textsuperscript{9,28} Although chylomicrons were also relatively enriched in 18:1, they contained reduced levels of vitamin E and no measurable PAF-AH activity. The absence of these 2 latter components may have rendered them more susceptible to oxidation, thus permitting chylomicrons to more readily develop bioactivity (compared with VLDL) when mildly oxidized.

We also now report that when conditioned on 15-LO fibroblasts, dense LDL developed more bioactivity compared with buoyant LDL. We, and others, have previously reported that dense LDL has greater susceptibility to copper-mediated oxidation compared with buoyant LDL.\textsuperscript{10,11} These present observations support the hypothesis that dense LDL is more atherogenic than buoyant LDL.\textsuperscript{35,36,52} In contrast, chylomicron size did appear to influence its susceptibility to oxidation in the coculture system. Whereas this fraction was readily modified by the 15-LO fibroblasts, this was not the case when it was added to the coculture system. Because the coculture uses lipoprotein-deficient serum, which contains sufficient antioxidants to inhibit oxidation in most cases, it has been presumed that lipoprotein modification within the coculture systems requires entry into the proteoglycan-rich matrix between the 2 cell layers.\textsuperscript{4,5} In this microenvironment, relatively “free” from antioxidants, lipoprotein oxidation can occur. Presumably, chylomicrons are too large to enter the subendothelial space and therefore cannot become minimally modified. This finding is consistent with the reports by Nordestgaard and Zilversmit\textsuperscript{53} and Van Heek and Zilversmit\textsuperscript{54} who demonstrated in animal models that elevations in chylomicrons do not enhance atherosclerosis in rabbits.

This study demonstrates that all ApoB-containing lipoproteins acquire proinflammatory activity when minimally oxidized. Factors that can modulate the degree of bioactivity that develops include lipoprotein fatty acid composition, PAF-AH content, and lipoprotein size. These data also support the concept that triglyceride-rich lipoproteins may also be proatherogenic.

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