Liposomes enriched in oleic acid are less susceptible to oxidation and have less proinflammatory activity when exposed to oxidizing conditions

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Abstract **As there is frequently a reciprocal relationship between oleic acid and linoleic acid content in LDL after dietary supplementation, it is difficult to determine the independent effects of oleic and linoleic acid on LDL oxidation. It is also unknown whether monounsaturated fatty acid enrichment might reduce the generation of proinflammatory products that occur when the polyunsaturated fatty acid-rich phospholipids within lipoproteins undergo mild oxidation. To address these issues, we exposed liposomes containing variable amounts of oleic, linoleic, and arachidonic acid to oxidizing conditions. Liposomes enriched in oleic acid but with constant amounts of linoleic acid were less susceptible to oxidation and had significantly greater lag times and time to half maximum conjugated diene formation. When mildly oxidized, liposomes containing either linoleic acid or arachadonic acid increased monocyte chemotaxis and monocyte adhesion to endothelial cells nearly 5-fold, demonstrating that oxidation products of both these polyunsaturated fatty acids are bioactive. The addition of a platelet activating factor receptor antagonist to endothelial cells inhibited stimulation of monocyte adhesion by oxidized liposomes, suggesting that some bioactive oxidation products of polyunsaturated fatty acids may resemble platelet activating factor in structure. In contrast, when liposomes were enriched in oleic acid, monocyte chemotaxis and monocyte adhesion were nearly completely inhibited. These results suggest that enriching lipoproteins with oleic acid may reduce oxidation both by a direct "antioxidant"-like effect and by reducing the amount of linoleic acid available for oxidation as well as reduce the generation of bioactive particles that occur during mild oxidation.—**Lee, C., J. Barnett, and P. D. Reaven. **Liposomes enriched in oleic acid are less susceptible to oxidation and have less proinflammatory activity when exposed to oxidizing conditions. *J. Lipid Res.* 1998.** 39: **1239–1247.**

Supplementary key words monounsaturated fatty acids • polyunsaturated fatty acids • *trans* fatty acids • vitamin E • platelet activating factor • lipid peroxidation • monocyte adhesion • chemotaxis • minimally modified low density lipoprotein

Many lines of evidence suggest that oxidation of low density lipoproteins (LDL) may play an important role in early atherosclerotic lesion formation (1–4). This has resulted in attempts to better understand the factors that influence LDL oxidation. Because oxidation of LDL begins with the abstraction of hydrogen from polyunsaturated fatty acids, it seemed quite probable that the polyunsaturated fatty acid content in LDL would play an important role in its susceptibility to oxidation. Parthasarathy et al. (5) first demonstrated this in New Zealand white rabbits fed a linoleate-enriched diet for 6 weeks. LDL isolated from these animals was both more readily and more extensively oxidized when exposed to oxidative stress in vitro. We and others subsequently demonstrated in humans that diets enriched in linoleate (18:2), compared to diets enriched in oleate (18:1), led to plasma lipoproteins that were enriched in 18:2 and were more susceptible to oxidation (6–10).

Although there is an overall consistency of results among these studies, several issues remain unclear. First, as the content of 18:2 is reduced in LDL when subjects are placed on oleate-enriched diets, there is frequently a reciprocal rise in the content of 18:1 in the LDL. Therefore, it is not yet established whether the effects of dietary alterations on LDL oxidation result from decreases in 18:2 content in LDL, from increases in 18:1, or from both effects. Additionally, in some studies the replacement of 18:2 with 18:1 led to a delay in initiation of LDL oxidation (7, 8), while in others there was a reduction in the extent of oxidation or both events occurred (10). Thus, the overall effect of replacing polyunsaturated fatty acids with monounsaturated fatty acids on lipid oxidation also remains unclear. Although it has been demonstrated that nonesterified monounsaturated fatty acids may inhibit transition metal-mediated lipid peroxidation (11), it is un-

Abbreviations: 18:1, oleic acid; 18:2, linoleic acid; mm-LDL, minimally modified low density lipoprotein; PC, phosphatidylcholine; $NaCl^{-/-}$, sodium chloride; AAPH, 2,2-azobis (2-amidinopropane) dihydrochloride; 15-LO, 15-lipoxygenase; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid reactive substances.

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known whether monounsaturated fatty acids incorporated into phospholipids or other lipid fractions have similar "antioxidant" effects.

Recently it has become clear that the extent of LDL oxidation may determine which of a multitude of potential atherogenic properties of oxidized LDL predominate at a given point in time. When LDL is less oxidized, i.e., minimally modified LDL (mm-LDL), it stimulates monocyte chemotaxis, transmigration, and adherence to endothelial cells (2, 12–15). Minimally modified LDL also stimulates expression of a number of growth factors such as M-CSF (15, 16). These and other properties of mm-LDL are undoubtedly important in initiating the earliest steps of atherosclerosis. Recent work has indicated that several of the proinflammatory properties of mm-LDL may result from oxidized phospholipids. Watson et al. (17) have characterized several of these oxidized phospholipids and believe that at least one group of proinflammatory compounds results from oxidative degradation of arachidonic acid within the phospholipid. However, the possibility that oxidation of other long chain fatty acids may generate bioactive compounds also needs evaluation.

The current studies use phospholipid liposome preparations to more specifically address the direct effects of monounsaturated and polyunsaturated fatty acids on lipid oxidation in liposomes. The results indicate that oxidation of both linoleic and arachidonic fatty acids within liposomes generate bioactive particles that stimulate monocyte chemotaxis and adhesion to endothelial cells. These studies also demonstrate that increasing the content of 18:1 in polyunsaturated fatty acid-rich liposomes has a direct "antioxidant"-like effect, similar to that of vitamin E. Furthermore, only a modest level of liposome enrichment with 18:1 is necessary to inhibit the bioactivity of oxidized liposomes.

METHODS

Materials

Synthetic phospholipids were obtained from Avanti-Polar Lipids, Inc. (Alabaster, AL) and included 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine, 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine, 1,2 diarachidonoyl-*sn*-glycero-3-phosphocholine. All phosphocholines were stored in chloroform, under argon at −20°C. Saturated and monounsaturated phosphocholines were free of added antioxidants, while polyunsaturated phosphocholines had less than 1 ng/ml of BHT, as determined by gas chromatography mass spectrometry (Avanti-Polar Lipids, Inc.)

Liposomes

Liposomes were composed of phosphatidylcholine (PC) and cholesterol at a 2:1 molar ratio. A variety of different phosphatidylcholines, which in some cases contained different fatty acids in the first or second position, were utilized for liposome preparation. Cholesterol and PC were combined and gently dried under nitrogen. The dried lipids were resuspended in a 40 mmol/L phosphate-buffered saline/140 mmol/L NaCl solution at a final total lipid concentration of 1.5 mmol/L, and extruded repeatedly through a Lipex Rapid Extruder (Lipex Biomembranes, Inc, Vancouver, B.C.) with two stacked polycarbonate 100-nm filters under 250 pounds of nitrogen pressure until clear (approximately ten times). Recovery of phospholipid was greater than 85%. The liposomes were used immediately after preparation. This procedure produces mixtures of unilamellar and multilamellar vesicles of just greater than 100 nm in size. Liposome size was determined by a laser particle sizer (Nicomp Model 370, Hiac/Royco Instruments).

Liposome oxidation

The formation of conjugated dienes was measured by incubating liposomes at 100 μg PC/ml with 10 μmol/L copper sulfate in 1 ml of PBS at 30°C. In some experiments liposome oxidation was compared in conjugated diene assays that were performed at different temperatures (23°C, 30°C, or 37°C). The absorbance at 234 nm, and in some cases from 200–300 nm, was measured continuously in a Uvikon 951 spectrophotometer. In additional experiments, oxidation was initiated with 150 mmol/L AAPH rather than with copper sulfate. 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) was determined. The maximum level of conjugated diene formation and the time to half maximum value ($T^{1/2}$max) were also determined as well. In some experiments the extent of copper-mediated lipid oxidation was determined by the measurement of lipid peroxides formed (18), or by the generation of malondialdehyde products (TBARS assay) using the method of Yagi (19), or by the loss of polyunsaturated fatty acid content (10). In some instances liposomes were oxidized by exposure to mouse peritoneal macrophages for 20 h in F-10 media (7).

Fatty acid determinations

Lipids from liposome samples were extracted by a modification of the method of Folch, Lees, and Sloane Stanley (20). 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 previously described (7). In some instances, prior to fatty acid determinations, liposomes were passed through Sepharose 4B columns to ensure separation of liposomes from unincorporated phosholipids and cholesterol.

Cell culture procedures

Resident mouse macrophages were harvested from the peritoneal cavity by lavage and plated on 24-well dishes at a density of 1.4×10^6 cells per well with RPMI medium containing 10% fetal calf serum as previously described (7). After an overnight incubation, the medium was replaced with serum-free medium and liposomes were added for up to 20 h. The supernatant was then removed for measurements of lipid peroxidation.

Murine fibroblasts expressing high levels of intracellular 15-lipoxygenase (15-LO) were established by infection with a retroviral vector as previously described (21, 22) and were used between passages 10–15. It has been previously demonstrated that in contrast to control fibroblasts expressing β-galactosidase, incubation of LDL on these 15-LO cells generates mm-LDL that can stimulate monocyte chemotaxis and adhesion to endothelial cells (23). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose (10 mmol/L), containing 10% fetal calf serum (FCS) and G418 sulfate (50 mg/ml) at 37°C and in 5% CO_2. Fibroblasts were plated on 96-well plates at 35,000 cells/well and grown for 2 days until nearly confluent. The cells were washed free of serum, and liposomes (200 μg of PC/ml) were then incubated with the fibroblasts at 37°C for 20 h in Hams

F-10 media. Liposome oxidation was determined by levels of TBARS formed and bioactivity was assessed as described below.

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 Co.). The cells were then plated in RPMI 1640 (Biowhittaker) + 10% homologous serum for 3 h at 37°C. Non-adherent cells were washed off and the adherent monocytes were released using PBS containing 0.18% EDTA and were then washed twice in PBS (24). Monocytes were then frozen in 10% DMSO, 30% serum, and 60% RPMI media and stored in liquid nitrogen until used.

Monocyte chemotaxis assay

Assays were performed in chemotaxis chambers (Neuro Probe Inc, Cabin John, MD) with a polycarbonate filter (Poretics, Livermore, CA) of 5 μm pore size separating the upper and lower chambers. The lower wells were filled with 28 μl of supernatant (diluted 1:5 in 0.1% BSA Tyrode's salt solution) from the fibroblast incubation experiments and approximately 100,000 monocytes were added to the top wells, and then the chambers were treated as previously described by Berliner, Territo, and Sevanian (25). The monocytes that migrated from the upper chamber to the lower surface of the filter were then counted using a light microscope and expressed as cells per high power field. The results of at least 4–8 wells were averaged for each experimental condition.

Monocyte adhesion assay

The assay, with minimal modifications, was carried out as described by Navab et al. (14). Liposomes conditioned in F-10 media alone or by 15-LO overexpressing fibroblasts in F-10 media for 20 h were transferred to confluent porcine aortic endothelial monolayers in 96-well tissue culture plates and the plates were incubated for 4 h at 37°C. After the 4-h incubation, the supernatant was removed and the endothelial monolayers were washed twice with RPMI 1640. THP-1 cells (a monocyte-like cell line) were placed on the endothelial cells at 45,000 cells/well, and the plates were incubated for 20 min at 37°C. The suspension was removed, and the cells were vigorously washed (at least 3 times) to remove all but the firmly adherent THP-1 cells. The number of adherent THP-1 cells was determined in four high power fields per well and the results of 4–8 separate wells were averaged for each experiment. In some experiments the platelet activating factor receptor antagonist Lau 603 (26) was added to the endothelial cells just prior to the addition of the 15-LO cell supernatant.

Statistics

One way ANOVA was used to compare values amoung multiple groups and post hoc comparisons between specific groups were performed by Student's unpaired *t* test. Data are presented as mean ± SD in tables and mean ± SE in graphs.

RESULTS

Liposomes consisted of free cholesterol and synthetic phosphatidylcholines containing palmitic, oleic, linoleic, or arachidonic acid. In the standard liposome preparations the PC:cholesterol ratio was 2:1. However, in some experiments cholesterol-free liposomes were evaluated to insure that cholesterol or products of cholesterol oxidation were not influencing results. When particle size was measured shortly after liposome preparation, there was consistently only one major peak of relatively uniform size in the range of 110–130 nm. Over time however, minor peaks of larger size developed. Liposome size was not influenced by the type of phospholipid used in liposome preparation.

The fatty acid composition of the prepared liposomes reflected very closely the intended composition. As shown in **Table 1**, we were able to increase the content of 18:1 in the liposomes while maintaining the content of 18:2 constant. In a similar fashion, we were able to increase the 18:2 or 20:4 content in the liposomes while keeping the 18:1 content steady (data not shown). In this way we were able to prepare liposomes in which the content of monounsaturated or polyunsaturated fatty acids was increased independently of each other. The content of polyunsaturated fatty acids in liposomes, as determined by gas chromatography, did not decrease during liposome preparation, indicating that lipid oxidation during the process was not a significant problem.

Our initial oxidation experiments demonstrated that liposomes containing 18:1, but little or no 18:2, showed little oxidation as measured by TBARS, conjugated diene formation, or loss of fatty acid content. Therefore, all subsequent experiments, were performed with at least a moderate amount of the fatty acids in the liposome in the form of 18:2 unless otherwise stated. As shown in **Fig. 1A**, as the content of 18:2 in the liposome was increased, so was the peak amount of conjugated dienes formed when the liposomes were oxidized by exposure to copper sulfate. In general, neither the lag time nor $T^{1/2}$ max varied substantially as a result of the increasing amounts of 18:2. In occasional experiments, however, oxidation of liposomes containing greater levels of 18:2 generated shorter lag times as well as the expected higher peak level of conjugated diene formation. This appeared related to the age of the 1,2 dilinoleoyl-*sn*-glycero-3-phosphocholine used to prepare the liposomes, presumably a result of PC oxidation occurring during storage. For this reason we routinely measured the fatty acid composition of the liposomes used in experiments to ensure that variation in 18:2 content did not contribute to differences in rates of liposome oxidation.

When 16:0 in the liposome was replaced in part by 18:1, while keeping 18:2 constant, there was a marked increase in the lag time of conjugated diene formation, demon-

TABLE 1. Fatty acid composition of prepared liposomes

Fatty Acid	16:0	18:1	18:2
	percent of total fatty acids		
Desired	62.5	0.0	37.5
Actual	57.1 ± 8.0	0.4 ± 0.9	39.0 ± 6.1
Desired	37.5	25.0	37.5
Actual	32.4 ± 4.9	29.0 ± 2.5	37.9 ± 3.0
Desired	25.0	37.5	37.5
Actual	22.4 ± 1.0	37.6 ± 2.3	37.7 ± 5.2
Desired	12.5	50.0	37.5
Actual	14.0 ± 0.8	50.8 ± 3.2	35.3 ± 3.5

Shown are the mean values ± SD for 16:0, 18:1, and 18:2 contents of the prepared liposomes, expressed as a percent of total fatty acids. Results represent the averages of 3–8 different liposome preparations at each selected distribution of fatty acids.

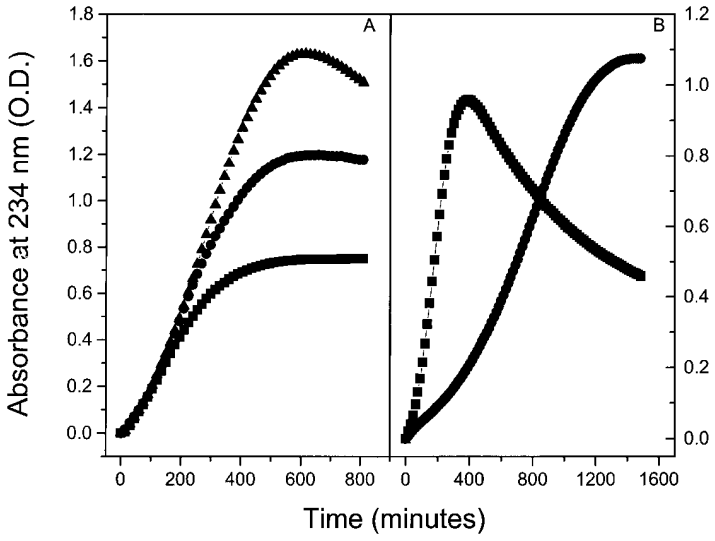

Fig. 1. Conjugated diene formation during copper-mediated oxidation of liposomes. In panel A, liposomes consisting of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine as a source of 16:0 and increasing amounts of 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine as a source of 18:2 (to achieve 25% (■), 50% (●), and 75% (▲) of the total fatty acids in the liposome) were oxidized by exposure to copper sulfate at 10 μmol/L and absorbance was recorded at 234 nm. In panel B, the 18:2 content within the liposomes was kept constant at 37.5% of the total fatty acids, while 1,2-dioleoyl-*sn*-glycero-3-phosphocholine was increased as a source of 18:1 (■ 0%, ● 50%), while decreasing the content of 16:0. Shown is one set of representative experiments.

strating inhibition of liposome oxidation (Fig. 1B). The same peak level of conjugated diene formation was eventually reached, however, suggesting that at these moderate amounts of 18:1 replacement, the oxidation reaction was delayed but not inhibited completely. To more closely examine this "antioxidant like" property of 18:1, we prepared liposomes in which the content of 18:1 was increased in steps from 0 to 50% of the total fatty acids, while maintaining 18:2 content constant at 37.5%, and then oxidized the liposomes by incubation with copper. As shown in **Table 2**, as 18:1 content within the liposome increased so did the lag time and the time to $T^{1/2}$ max. Although the absolute lag times and times to $T^{1/2}$ max varied for each liposome preparation, the protective effect of 18:1 was consistent. Similar results were obtained with pure PC liposomes, i.e., not containing any cholesterol (data not shown). To ensure that changes in fatty acid content did not invalidate measuring absorbance at 234 nm as a method to detect conjugated diene formation during liposome oxidation, we also recorded absorbance from 200–300 nm during the initial 7 h of copper-mediated oxidation. Increasing the amount of 18:1 in the liposomes slowed the increase in absorbance at 234 nm over this time frame, but did not change the wavelength of maximum absorbance (data not shown). Oleic acid enrichment still slowed oxidation when the liposome 18:2 content was replaced with 20:4 or mixtures of both 18:2 and 20:4. This "antioxidant" effect of 18:1 persisted in liposomes in which enrichment with 18:1 was generated by using phospholipids with 18:1 in the *sn*1, *sn*2, or both positions (data not shown).

TABLE 2. Effect of increasing 18:1 content on oxidation of liposomes

Percent 18:1	Lag Time	$T^{1/2}$max	Peak Abs
	min	*min*	*O.D*
0	57.4 ± 23.2	186.8 ± 43.9	0.91 ± 0.1
25	178.4 ± 49.2[a]	401.4 ± 123.8[a]	0.96 ± 0.15
50	230.5 ± 105.2[a]	617.8 ± 188.4[a]	0.94 ± 0.13

Shown are lag times, time to reach half-maximum peak conjugated diene formation ($T^{1/2}$max), and peak absorbance (O.D units) of conjugated diene formation obtained for liposomes with increasing amounts of 18:1. Samples were oxidized with 10 μmol/L copper sulfate as described in Methods. Data represent the mean values ± SD of 4 experiments.

[a] Significant at $P < 0.05$ compared to liposomes without 18:1.

To demonstrate that these effects of 18:1 were observed under other conditions of oxidation we performed several additional experiments. We demonstrated that increasing the content of 18:1 in liposomes led to an increase in the lag time regardless of whether the conjugated diene assay was performed at 23°C, 30°C or 37°C, or in the presence of much higher concentrations of copper (data not shown). As shown in **Fig. 2**, enriching liposomes with 18:1 inhibited oxidation as assessed by the delay in formation of lipid peroxides (panel B), which paralleled very closely the delay in conjugated diene formation (panel A). This effect of 18:1 was not dependent on a copper-mediated system of oxidation. As shown in panel C, similar effects on lipid oxidation were seen when liposomes were oxidized with AAPH, a water-soluble free radical generator. Furthermore, liposomes enriched with 18:1 were also less susceptible to oxidation mediated by mouse peritoneal macrophages (panel D).

The liposome model allowed us to evaluate several other relevant issues of lipid oxidation. There is little published information on whether adding antioxidants to lipid-containing particles that are already enriched in oleic acid significantly improves antioxidant protection. In the presence of increasing amounts of vitamin E in the reaction mixture, copper-mediated oxidation of liposomes was markedly inhibited, resulting in progressive lengthening of the lag time (**Fig. 3A**). When liposomes that were enriched with 18:1 while 18:2 levels were kept constant were oxidized in the presence of vitamin E, they had a substantially greater delay in oxidation than did the same liposomes oxidized in the absence of vitamin E (Fig. 3B) suggesting at least an additive resistance to oxidation when liposomes are enriched in both vitamin E and 18:1.

Recent studies have suggested that dietary intake of *trans*-monounsaturated fatty acids may be proatherogenic

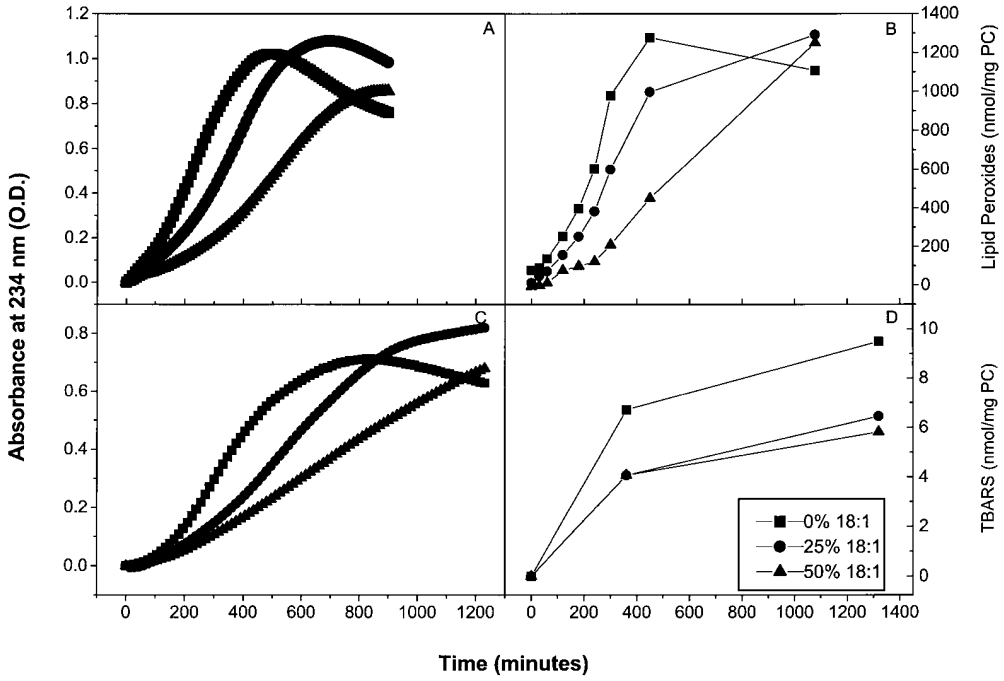

Fig. 2. Oxidation of 18:1 enriched liposomes as measured by different assays of lipid peroxidation and after exposure to different types of oxidant stress. Shown in each panel are liposomes containing 0 (■), 25% (●) or 50% (▲) 18:1. In panel A, liposomes was exposed to 10 μmol/L copper sulfate and oxidation are assessed by conjugated diene formation. In panel B, liposomes were exposed to 10 μmol/L copper sulfate and oxidation was assessed by formation of lipid peroxides (18). In panel C, liposomes were exposed to 150 mmol/L AAPH and oxidation was assessed by conjugated diene formation. In panel D, liposomes were exposed to peritoneal macrophages for 16 h and oxidation was followed by generation of TBARS. Shown is one set of representative experiments.

as a result of their tendency to elevate plasma cholesterol levels. To assess whether *trans*-monounsaturated fatty acids may also have a unique influence on lipid oxidation, we prepared liposomes in which 0%, 25%, or 50% of their total fatty acids were in the form of 18:1 *cis*- or *trans*-fatty acids. At each concentration, the 18:2 content within the liposomes was kept constant at 37%. Gas chromatographic analysis of liposome fatty acid content demonstrated that equal contents of *trans*- and *cis*-18:1 were incorporated within the liposomes. Liposomes containing *trans*-monounsaturated fatty acids demonstrated a delay in oxidation that was similar to that of liposomes containing *cis*-monounsaturated fatty acids (data not shown). Similar effects were seen when other monounsaturated fatty acids, e.g., 16:1, were incorporated into liposomes.

Although there is increasing evidence that mm-LDL may be an important proinflammatory agent in the artery wall, little is known about the products of LDL oxidation that induce bioactivity. Recent studies have tested the oxidation products of individual fatty acids for bioac-

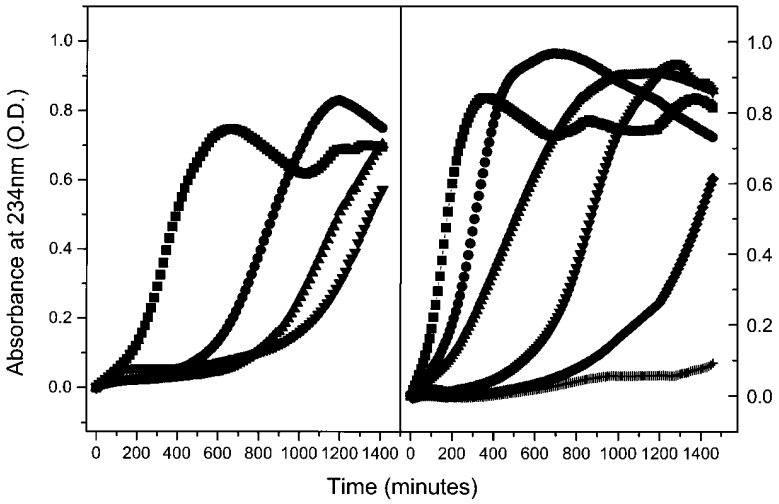

Fig. 3. The effect of vitamin E on liposome oxidation. In panel A, liposomes containing 62.5% 16:0 and 37.5% 18:2 were exposed to 10 μmol/L copper sulfate in the presence of increasing amounts of vitamin E (0 (■), 6.25 (●), 12.5 (▲) and 25 (▼) μmol/L) in the media. In panel B, liposomes with increasing amounts of enrichment with 18:1 (0% (■), 25% (●) and 50% (▲)) were oxidized in the absence or presence of 6.25 μmol/L vitamin E (0% 18:1 + vit E (▼), 25% 18:1 + vit E (◆), 50% 18:1 + vit E (†)). Shown is one representative experiment of a total of three performed.

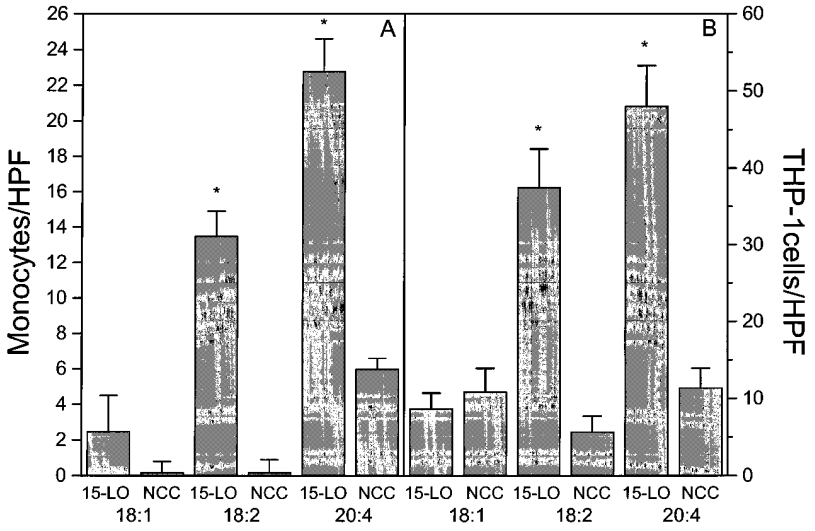

Fig. 4. Stimulation of monocyte chemotaxis and adherence of THP-1 cells to endothelial cells by liposomes modified on 15-LO cells. Liposomes containing 50% 16:0 and 50% 18:1, 18:2 or 20:4 were incubated on 15-LO cells or in F-10 media alone (NCC) for 20 h and then the supernatant was used for determination of monocyte chemotaxis activity or stimulation of THP-1 cell adhesion to endothelial cells as described in Methods. For chemotaxis assays, the lower wells of the chambers were filled with 28 μl of supernatant (diluted 1:5 in 0.1% BSA Tyrode's salt solution) and the chambers were treated as previously described by Berliner et al. (25). The monocytes that migrated from the upper chamber to the lower surface of the filter were then counted using a light microscope and expressed as cells per high power field. The results of 4–8 wells were averaged for each experimental condition and shown in panel A are the means ± SE of three separate experiments. For adhesion assays, the supernatant was incubated with endothelial cells for 4 h. After repeated washing of the endothelial cells, THP-1 cells were added to the endothelial cells for 20 min then rewashed and the remaining adherent cells were counted. Shown in panel B are the means ± SE of the adherent cells/per high power field of three separate adhesion experiments. *Significant compared to NCC values.

tivity and have suggested an important role for products of oxidized arachidonoyl-*sn*-glycero-3-phosphocholine (27). However, other polyunsaturated fatty acids may also generate proinflammatory products when oxidized. Incubation of liposomes containing 16:0 along with either 18:2 or 20:4 with fibroblasts overexpressing 15-LO induced modest oxidation of the liposomes (TBARS 15–35 nmol/mg PC). However, these liposomes were able to stimulate increased monocyte chemotaxis and monocyte adhesion to endothelial cells (**Fig. 4**). These events were inhibited by the presence of transition metal chelators or antioxidants such as EDTA or BHT, respectively, during the conditioning of the liposomes on the fibroblasts. By testing both lipids extracted from the 15-LO cell-conditioned liposomes as well as the liposome-free cell supernatant (separated from the liposomes by passing the aqueous incubation mixture through a membrane cone (Amicon, Beverly, MA)) we were able to demonstrate that the bioactive products of these cell-conditioned liposomes resided primarily within the lipids (data not shown). In contrast, neither liposomes that contained 18:1 in place of polyunsaturated fatty acids (Fig. 4) nor media alone were able to generate bioactivity when conditioned on 15-LO cells (data not shown). Enriching the liposomes with as little as 12.5 % of 18:1 decreased their ability to stimulate chemotaxis and monocyte adhesion to endothelial cells by greater than 70% (**Fig. 5**). Interestingly, TBARS generated during liposome modification on 15-LO cells were only modestly reduced in liposomes with increased 18:1 content (**Table 3**). Liposomes enriched with 18:1 but cholesterol-free also reduced monocyte chemotaxis and stimulation of monocyte adhesion to endothelial cells (data not shown). These data suggest that at least under these experimental conditions, oxidation products of both 18:2 and 20:4 have proinflammatory properties.

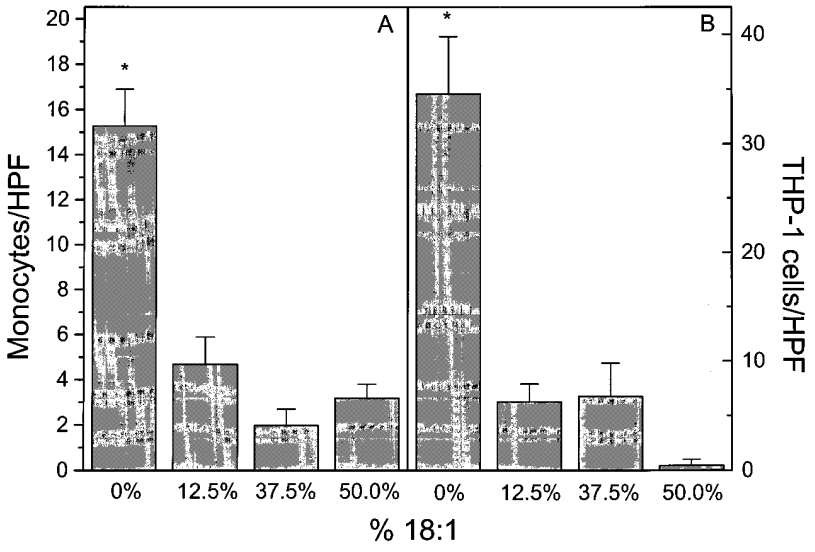

Fig. 5. Inhibition of monocyte chemotaxis and adherence of THP-1 cells to endothelial cells by 18:1-enriched liposomes. 1,2-Dilinoleoyl-*sn*-glycero-3-phosphocholine as a source of 18:2 was kept constant at 37.5% of total liposome fatty acids while 1,2-dioleoyl-*sn*-glycero-3-phosphocholine was increased as a source of 18:1, while decreasing the content of 16:0. Liposomes containing 0%, 12.5%, 37.5%, and 50% 18:1 were incubated on 15-LO cells or in F-10 media alone (NCC) for 20 h and then the supernatant was used for determination of monocyte chemotaxis activity or stimulation of THP-1 cell adhesion to endothelial cells as described in Methods and in Fig. 4. The results of at least 4–8 wells were averaged (minus NCC values) for each experimental condition. Shown in panel A are the means ± SE of three separate chemotaxis experiments while shown in panel B are the means ± SE of three separate adhesion experiments. *Significant compared to 18:1 enriched liposomes.

TABLE 3. Oxidation of 18:1-enriched liposomes

18:1 within the Liposome	TBARS: Incubation on 15-LO Cells	TBARS: Incubation in Media
%	*nmol MDA/mg PC*	
0	18.0 ± 3.0	12.5 ± 1.0
12.5	17.9 ± 2.7	11.9 ± 0.7
37.5	16.1 ± 3.1	9.2 ± 1.7
50.0	14.6 ± 3.3	8.4 ± 1.9

Liposomes with 37.5% of their total fatty acids in the form of 18:2 and increasing amounts of 18:1 with corresponding decreases in the amount of 16:0 were prepared as described in Methods. Liposomes were conditioned on either 15-LO cells or in media alone for 20 h. TBARS were measured on the supernatant and are presented as the means ± SD of three experiments.

Although the identity of these proinflammatory products remains unknown, some of these products may resemble platelet activating factor and act in part through the platelet activating factor receptor, as previously suggested (23, 28, 29). Addition of the platelet activating factor receptor antagonist Lau 603 to endothelial cells nearly completely blocked the induction of monocyte adhesion by 15-LO conditioned liposomes (**Fig. 6**). Neither platelet activating factor receptor antagonist alone nor its vehicle nor platelet activating factor receptor antagonist added in the presence of LPS had inhibitory effects on monocyte adhesion. Furthermore, under these conditions, lysophosphatidylcholine could not be detected in the supernatant containing cell-conditioned liposomes (data not shown).

DISCUSSION

A major point of this study was to evaluate the individual effects of oleic and linoleic acids on lipid peroxidation to gain better insight into their respective roles in lipoprotein oxidation. Utilization of the liposome model offered several advantages. Foremost among these was the ability to independently alter the content of either oleic or linoleic acid within the liposomes. Additionally, liposomes composed of phosphatidylcholine and cholesterol have structural similarities to the phospholipid-rich exterior of the LDL particle, although they do not contain the many different protein moieties present within LDL.

Liposomes enriched in 18:2 generated greater conjugated dienes when oxidized, consistent with the greater amount of substrate available for oxidation. The lag time and rate of oxidation did not appear to vary much as 18:2 was increased above 25% of total fatty acids (Fig. 1A), consistent with prior in vitro studies (30, 31). In contrast, enriching liposomes with 18:1, while keeping 18:2 constant (both total mass and percent of total fatty acids), lengthened the lag time, but had little effect on the absolute level of conjugated diene formation unless 18:1 was present at sufficiently high levels to completely suppress oxidation. Oxidation of 18:1-enriched liposomes was reportedly reduced in studies by Vossen et al. (32), although the content of 18:2 within these liposomes was modestly decreased. This effect of increased 18:1 within liposomes suggests that in certain situations 18:1 may have "antioxidant" type effects on lipid peroxidation. In support of this concept, Balasubramanian, Nalini, and Manohar (33), using fatty acids bound to albumin, showed that oxidation of liver microsomes could be inhibited by the addition of unesterified fatty acids. Fatty acids with a single double bond, such as oleic acid, were particularly effective inhibitors of oxidation. In several human studies in which diets were enriched in 18:1, the delay or lag time prior to rapid oxidation of the isolated LDL was increased, consistent with an "antioxidant" effect (7, 8). The mechanism by which 18:1 inhibits oxidation is not known. The studies by Balasubramanian et al. (11, 33) suggested that much, but not all, of the inhibition of oxidation by unesterified monounsaturated fatty acids could apparently be explained by iron chelation. However, this is not a viable mechanism for protection of liposomes comprised of phospholipids. Moreover, in the current studies, 18:1 still provided "antioxidant activity" in non-transition metal-mediated systems of oxidation. An alternative explanation may be that 18:1 may act as a "competitive inhibitor" of oxidation of polyunsaturated fatty acids. With one double bond it can undergo oxidation but its rate of oxidation is much less than that of polyunsaturated fatty acids (34). It has been suggested that the monohydroxy products generated during oxidation of 18:1 or other monounsaturated fatty acids may be more stable and less

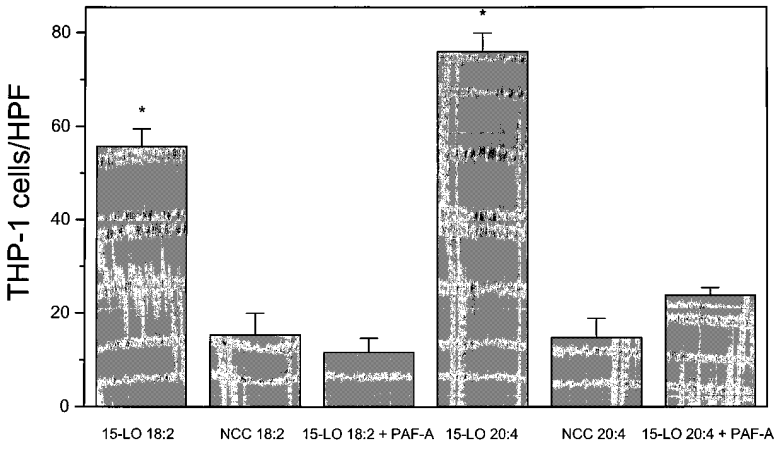

Fig. 6. Inhibition of liposome stimulated monocyte adherence of THP-1 cells to endothelial cells by a platelet activating factor receptor antagonist. Liposomes containing 50% 16:0 and 50% 18:2 or 20:4 were incubated on 15-LO cells or in F-10 media alone (NCC) for 20 h and then the supernatant was used to stimulate THP-1 cell adhesion to endothelial cells as described in Methods. In some wells the platelet activating factor receptor antagonist Lau 603 was added to the endothelial cells just prior to the addition of the conditioned liposomes. The results of at least 4–8 wells were averaged for each experimental condition and shown are the means ± SE of the adherent cells/per high power field of four separate experiments. *Significant compared to no cell control values (NCC) and platelet activating factor receptor antagonist treated samples.

capable of further propagating oxidation of neighboring fatty acids (34). This is consistent with our finding that similar effects were seen with other monounsaturated fatty acids such as 16:1 or *trans*-18:1. Thus, when strategically placed they may still bind available oxygen, but have less impact on subsequent oxidation. In this fashion, oleic acid may act similarly to antioxidants like vitamin C or E (though by a different mechanism). These studies do not, however, allow direct comparisons of relative "antioxidant" activity among these different substances.

Using this liposome system provided us the opportunity to evaluate several other issues related to LDL fatty acid composition and LDL peroxidation. In several studies, the relationship of vitamin E to polyunsaturated fatty acid or monounsaturated fatty acid content in LDL has appeared particularly important in determining the susceptibility of LDL to oxidation. There has, however, been very little direct in vitro evaluation of these antioxidant/fatty acid interactions and human trials of combinations of monounsaturated fatty acid-enriched diets and antioxidants to reduce LDL oxidation have only rarely been tried (8, 35). Adding vitamin E to 18:1 enriched liposomes increased the resistance of these particles to oxidation. It appears that at least under these experimental conditions, this combination appears to have additive effects on inhibition of lipid oxidation. These data support further testing of the concept of combining diets enriched in 18:1 with antioxidants such as vitamin E in order to further reduce lipoprotein oxidation beyond that obtained by antioxidant supplementation alone.

The development of bioactivity when LDL undergoes early stages of oxidation is poorly understood but is likely to be an important aspect of atherogenesis. The current findings support the idea that oxidized phospholipids can enhance chemotaxis and stimulate endothelial cell expression of adhesion molecules. The exclusion of cholesterol from liposomes did not diminish the ability of liposomes conditioned on 15-LO cells to induce monocyte chemotaxis or monocyte adherence, thus demonstrating that oxidation products of cholesterol are not necessary for liposome bioactivity. Additionally, lysophosphatidylcholine was not formed during the oxidation of liposomes, presumably because liposomes do not contain the phospholipase needed for the cleavage of the *sn*2 fatty acid. Apparently, there is insufficient phospholipase activity expressed in murine fibroblasts to permit significant lysophosphatidylcholine formation in the conditioned liposomes.

As elegantly demonstrated by Watson et al. (17), oxidation products of phosphatidylcholine containing arachidonic acid appear particularly effective in stimulating monocyte adhesion. They have identified at least two of these compounds, 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (17), although there are likely many more bioactive particles formed. In our studies, oxidation of liposomes enriched with phosphatidylcholine containing linoleic acid also generated oxidation products capable of stimulating monocyte chemotaxis and monocyte adhesion. Moreover, the oxidation of phospholipid derived liposomes containing 18:2 as their only source of polyunsaturated fatty acids also generated products whose bioactivity could be inhibited by platelet activating factor receptor antagonists. This suggests that these oxidation products of phospholipids may resemble platelet activating factor, and may contain short chain oxidation products in the *sn2* position, similar to those identified by Watson et al. (17). However, the identification of these bioactive particles is currently unknown.

Given that linoleic acid is the predominant polyunsaturated fatty acid present in lipoproteins and cell membranes, gaining greater understanding of the structure and function of these oxidation products may have important implications for understanding and treating proinflammatory events in the artery wall. Importantly, even modest amounts of 18:1 in the liposomes dramatically decreased the bioactivity of 18:2-enriched liposomes. Of note, despite the marked decrease in bioactivity, TBARS were only slightly reduced as 18:1 content within the liposomes was increased (Table 3). This relative disassociation between TBARS levels and bioactivity has been consistently noted in 15-LO dependent systems of oxidation (23, 36). We previously speculated that 15-LO cells may induce generation of unique (or at least greater levels of) bioactive compounds in cell-conditioned LDL. Increasing 18:1 content in liposomes appears to inhibit this process without significantly reducing the overall extent of lipid oxidation as measured by TBARS.

When we extend the findings of this study to lipoproteins, they suggest that enriching diets in monounsaturated fatty acids may reduce oxidation both by a direct "antioxidant" like effect as well as by reducing the amount of 18:2 available for oxidation. Both effects may be important in reducing the amount of proinflammatory molecules generated during mild oxidation of LDL. The feasibility of this dietary approach has been demonstrated as we have previously shown that diets enriched in monounsaturated fatty acids increase the content of 18:1 and decrease the content of 18:2 in all lipid fractions of LDL (10). Furthermore, we have recently demonstrated that compared to individuals on normal diets, individuals who ingest diets enriched in monounsaturated fatty acids have LDL that are significantly less bioactive when exposed to oxidative stress (37).

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