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Essential role of phospholipase A_2 activity in endothelial cell-induced modification of low density lipoprotein

(atherosclerosis/foam cells/macrophages/lipid peroxides)

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ABSTRACT **Previous studies have established that incubation of low density lipoprotein (LDL) with cultured endothelial cells (EC) converts it to a new form (EC-modified LDL) that is now recognized by a specific receptor on macrophages (the acetyl LDL receptor) and is taken up and degraded 3–10 times more rapidly than native LDL (*biological modification*). The formation of EC-modified LDL depended on generation of free radicals with consequent peroxidation of LDL lipids and was accompanied by extensive hydrolysis of LDL phosphatidylcholine at the 2-position. The present studies show that *p*-bromophenacyl bromide, a site-specific irreversible inhibitor of phospholipase A_2 activity, blocks this hydrolysis and, at the same time, the enhanced macrophage degradation. We show further that during EC modification the apoprotein B of LDL undergoes considerable modification and that this also is prevented by the phospholipase inhibitor. Finally, as reported previously, changes similar to those observed on incubation of LDL with EC can be induced by incubation in the absence of cells but in the presence of a sufficiently high concentration of Cu^{2+}. This also is accompanied by hydrolysis of phosphatidylcholine at the 2-position and breakdown of apoprotein B. These changes are also inhibited by *p*-bromophenacyl bromide, suggesting the presence of a phospholipase A_2 activity associated with LDL as it is isolated. A hypothesis is presented linking lipid peroxidation, phosphatidylcholine hydrolysis, and changes in the LDL apoprotein during EC modification.**

Many or most of the lipid-laden foam cells of atherosclerotic lesions are derived from monocyte/macrophages (1–3). Yet, paradoxically, native low density lipoprotein (LDL) is recognized poorly by cultured macrophages and does not produce cholesterol ester accumulation in such cells in culture (4). Previously we have described a biological modification of LDL that converts it to a form recognized by macrophages and that leads to greatly enhanced cellular uptake and promotion of cholesterol ester accumulation (5–8). In part, the enhanced uptake is mediated via the "scavenger" receptor—the acetyl LDL receptor (4)—which also specifically recognizes several chemically modified forms of LDL (9, 10). The biological modification is effected by simply incubating native LDL overnight in the presence of cultured endothelial cells (EC) (5) or smooth muscle cells (7). The product, designated *EC-modified LDL* (EC-LDL), shows an increase in negative charge and hydrated density and is degraded *less* rapidly by the native LDL receptor (7). All of these changes were recently shown to be obligatorily linked to free radical-mediated peroxidation of LDL and to be accompanied by extensive hydrolysis of LDL phosphatidylcholine (PtdCho) to lyso-PtdCho (l-PtdCho) through apparent phospholipase A_2 activity (8). All of the compositional changes, as well as the *biological modification* (defined here as those changes that induce the increased rate of degradation of EC-LDL by macrophages), were blocked by antioxidants [vitamin E or butylated hydroxytoluene (BHT)]. They were also blocked by low concentrations of EDTA, concentrations sufficient to complex iron and copper in the medium but too low to significantly alter Ca^{2+} concentrations. It was concluded that initiation of EC modification depended on peroxidative changes catalyzed by metal ions. In the present paper we show that EC modification is associated also with extensive changes in apolipoprotein B (apo B). These protein changes, the hydrolysis of PtdCho, and most of the other changes that accompany EC modification, including the biological modification, are all blocked by *p*-bromophenacyl bromide (*p*BPB), an inhibitor of phospholipase A_2 activity. Thus, phospholipid breakdown is also an obligatory element in the series of changes involved in generating EC-LDL.

MATERIALS AND METHODS

Carrier-free $Na^{125}I$ and [1-^{14}C]linoleic acid were purchased from Amersham. Ham's F-10 medium was obtained from Irvine Scientific and Dulbecco's modified Eagle's medium was from GIBCO. Fetal bovine serum was supplied by Hyclone (Logan, UT). *p*BPB (2,4'-dibromoacetophenone) and soybean lipoxidase were purchased from Sigma and *p*-bromoacetophenone and acetophenone were from Aldrich. 1-Palmitoyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine was prepared as described (8). 1-Bromooctan-2-one was synthesized by reacting heptanoyl chloride with excess diazomethane, followed by treatment of the resulting diazoketone with 50% (wt/vol) aqueous hydrogen bromide (28).

Lipoproteins. LDL (ρ = 1.019–1.063) was isolated from fresh human plasma and radioiodinated as described (8). Unlabeled and labeled LDL were extensively dialyzed against phosphate-buffered saline containing 0.01% EDTA.

Cells. Rabbit EC, generously provided by V. Buonassissi, were grown in Ham's F-10 medium supplemented with 15% fetal bovine serum and EC modification of LDL was carried out as described (5). The modified LDL was used for degradation studies without reisolation. LDL was also modified in the absence of EC by incubating LDL (100 μg/ml) in 2 ml of Ham's F-10 medium containing 5 μM Cu^{2+} at 37°C for 24 hr (8).

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Abbreviations: LDL, low density lipoprotein; EC, endothelial cell(s); PtdCho, phosphatidylcholine; l-PtdCho, lyso-PtdCho; *p*BPB, *p*-bromophenacyl bromide; apo B, apolipoprotein B; B_{100}, the major form of apo B with $M_r \approx 549{,}000$; BHT, butylated hydroxytoluene.

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Resident peritoneal macrophages were harvested from female Swiss–Webster mice by peritoneal lavage and used in experiments after 24 hr in culture as described (7).

Degradation of ^{125}I-Labeled LDL (^{125}I-LDL). Medium containing ^{125}I-labeled lipoprotein samples was added to macrophages at 10 μg of LDL protein per ml in a total volume of 1 ml in serum-free F-10 or Dulbecco's modified Eagle's medium. After 5 hr of incubation at 37°C, the medium was removed and trichloroacetic acid-soluble, noniodide radioactivity was measured (8).

Lipid Peroxidation. The extent of lipid peroxidation was measured as thiobarbituric acid-reactive products and expressed as malondialdehyde equivalents (11). LDL (50 μg of protein) in 0.5 ml was mixed with 1.5 ml of 20% trichloroacetic acid and 1.5 ml of 0.67% thiobarbituric acid in 0.05 M NaOH. After heating in a boiling water bath for 30 min, the samples were centrifuged at 2000 rpm for 10 min and the optical density was read at 532 nm. Fresh tetramethoxypropane, which produces malondialdehyde, was used as standard.

Lipid extraction and analysis for lipid phosphorus were performed as described (8) and protein was determined by the method of Lowry *et al.* (12).

Preparation of Oxidized PtdCho. One micromole of 1-palmitoyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine, 5 μmol of sodium deoxycholate, and 0.2 mg of soybean lipoxidase (2×10^4 units) were incubated in 0.6 ml of 0.2 M borate buffer (pH 9.0). Fresh enzyme was added at 15-min intervals for 1 hr. The incubation mixture was extracted by the method of Bligh and Dyer (13) and the chloroform extract, containing 1-palmitoyl-2-[1-^{14}C]linoleoylhydroperoxy-*sn*-glycero-3-phosphocholine, was taken to dryness. Unlabeled linoleic acid, similarly oxidized, provided oxidized fatty acid as standard for TLC.

Oxidized or unoxidized PtdCho was used as the substrate for the assay of phospholipase activity in LDL. The incubation system contained 65 μM labeled PtdCho, 75 mM Tris (pH 7.4), 2.5 mM $CaCl_2$, 1 mM sodium deoxycholate, and 200 μM BHT, with or without 100 μg of LDL protein (total volume, 0.8 ml). Incubation was carried out at 37°C for 1 hr and the products were extracted by the method of Bligh and Dyer, with 1 ml of 1 M HCl in place of water. The chloroform layer was dried and lipids were separated by TLC on silica gel G plates by using $CHCl_3$/methanol/acetic acid/H_2O, 90:10:0.5:0.5 (vol/vol), as the solvent system. Lipids were identified after brief exposure to iodine, and the bands corresponding to linoleic acid and oxidized linoleic acid were scraped, eluted, and radioassayed. Appropriate control studies showed that recoveries from both unoxidized and oxidized substrates were equal and nearly quantitative. Apparent enzyme activities are expressed in terms of nmol of labeled free fatty acid released.

$NaDodSO_4$ Gradient Gel Electrophoresis. Lipoprotein samples were extracted by the method of Bligh and Dyer, stored at −20°C, and subsequently centrifuged in a fixed-angle rotor for 10 min at 2500 rpm at room temperature. The phases were removed carefully without disturbing the protein interface. The protein was then solubilized in sample buffer (containing 2% $NaDodSO_4$, 10% glycerol, and 5% 2-mercaptoethanol) by incubating in a boiling water bath for 3 min. Protein recoveries in experiments utilizing ^{125}I-LDL were found to be 70–105% of that in the medium before extraction. Electrophoresis was performed using 3–15% gradient gels or precast 2.5–27% gels (Isolab, Akron, OH) at a constant current of 25 mA for 5 hr using a Pharmacia vertical gel system. Gels were stained with Coomassie blue or with silver (14).

RESULTS

Effects of Inhibiting Phospholipase Activity on EC Modification of LDL. Previously we have reported that during EC modification of LDL there is extensive conversion of LDL PtdCho to l-PtdCho (as much as 40%) by an apparent phospholipase A_2 activity (8). To define the role of this activity we tested the effect of a potent inhibitor of phospholipase A_2, *p*BPB. As shown in Fig. 1, in the presence of *p*BPB at 25 μM the hydrolysis of PtdCho was significantly inhibited and at 40 μM it was completely prevented. The biological modification of LDL (its increased degradation by macrophages) was also inhibited and the degree of inhibition closely paralleled that of PtdCho hydrolysis. As shown in more detail in Fig. 2, LDL incubated in the presence of EC in the usual way was degraded by macrophages at a rate five times that for native LDL, whereas LDL incubated in the *absence* of cells (control LDL) was degraded at a rate no different than that for native LDL. This biological modification was progressively inhibited by *p*BPB and completely blocked at 40 μM. Addition of 40 μM *p*BPB to the LDL at the *end* of the incubation with EC did not affect subsequent macrophage degradation. At the higher concentrations, *p*BPB caused evident EC toxicity (detachment and rounding up of cells), which might have been the basis for the inhibition. However, EC *pretreated* overnight with 40 μM *p*BPB and then washed to remove the inhibitor were able to modify LDL in the usual manner (data not shown). In contrast, when the *LDL preparation* was preincubated with *p*BPB, excess *p*BPB was removed, and the treated LDL was then incubated with EC, biological modification was completely prevented (Table 1). These findings imply the presence of phospholipase activity associated with the LDL preparation, as suggested previously (8), and this has now been explicitly demonstrated as described below.

Since antioxidants can, as reported previously (8), block phospholipid hydrolysis and biological modification as well as peroxidation, we considered the possibility that the *p*BPB effect might be based on intrinsic antioxidant activity *rather* than its ability to inhibit phospholipases. Indeed, even at low concentrations of *p*BPB there was some apparent inhibition of lipid peroxidation, although this was much less than the inhibition of PtdCho hydrolysis and biological modification. Thus, as shown in Fig. 1, there was <10% inhibition of oxidation at 25 μM *p*BPB, whereas PtdCho hydrolysis and biological modification were inhibited by >40%. At 40 μM *p*BPB there was a clear inhibitory effect on peroxidation, although still much less than the *complete* inhibition of PtdCho hydrolysis and of biological modification (Fig. 1).

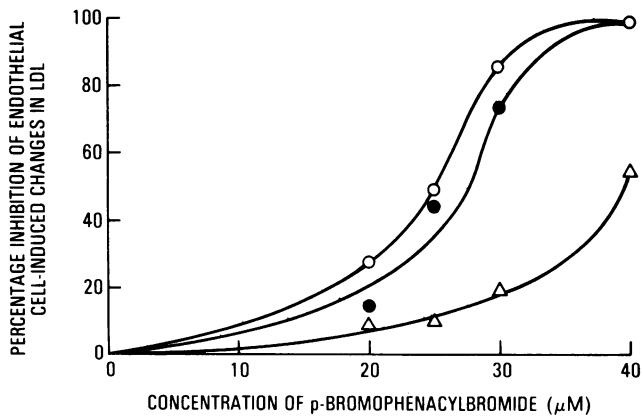

FIG. 1. Effect of *p*BPB on the formation of thiobarbituric acid-reactive materials (△), PtdCho hydrolysis (○), and macrophage degradation (●). ^{125}I-LDL (100 μg of protein per ml) was incubated with rabbit aortic EC for 24 hr at 37°C. *p*BPB was added at indicated concentrations at the beginning of the incubation in 10 μl of warm ethanol. At the end of incubation, medium was analyzed for PtdCho and l-PtdCho, thiobarbituric acid-reactive products, and macrophage degradation. Results are expressed as % change from unincubated LDL and are averages of at least three separate incubations.

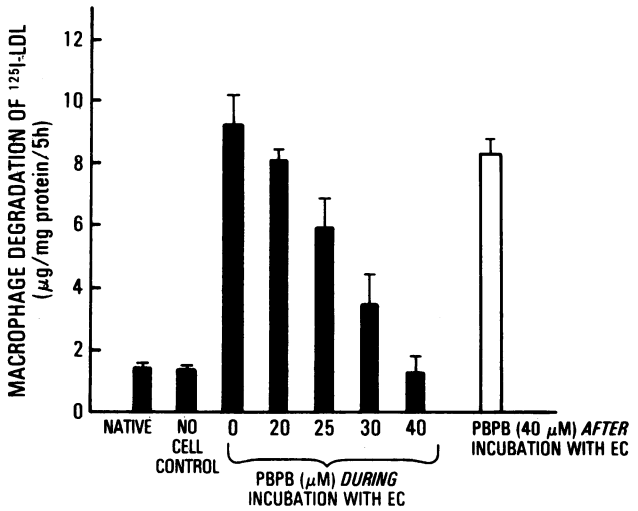

FIG. 2. Inhibition of EC modification of LDL by *p*BPB. ^{125}I-LDL (100 μg of protein per ml) was incubated with cultured rabbit aortic EC or in no-cell dishes at 37°C for 24 hr. *p*BPB, dissolved in warm ethanol, was added at the beginning (solid bars) or at the end of incubation (open bar) at the concentrations indicated in a 10-μl volume. The ^{125}I-LDL-containing medium was then transferred for measurement of degradation by cultured mouse peritoneal macrophages. Values are shown as mean ± SD of four separate incubations.

However, as shown in Table 2, two very closely related structural analogues of *p*BPB, differing only in the bromine substituents and not active as phospholipase inhibitors, were without effect on either peroxidation or biological modification. Similar results were obtained with 1-bromooctan-2-one, an aliphatic phospholipase inhibitor. At 80 μM this compound inhibited biological modification by 70% without any effect on the generation of thiobarbituric acid-reactive products. Thus, it is unlikely that *p*BPB acts *directly* as an antioxidant. There are, in theory, at least two ways in which the apparent decrease in generation of thiobarbituric acid-reactive material in the presence of *p*BPB might be indirect—i.e., secondary to its inhibition of phospholipase activity. First, *p*BPB may not really decrease the true amount of peroxidation. Instead, the color yield in the thiobarbituric acid assay may be less for ester-bound fatty acid peroxides than for free fatty acid peroxides. Second, phospholipase activity, by generating mobile free fatty acid peroxides, may facilitate propagation of lipid peroxidation through chain reactions; thus, *p*BPB would reduce the extent of peroxidation by preventing mobilization of these free fatty acid peroxides.

Table 1. Effect of preincubation of LDL with *p*BPB on biological modification by EC

Treatment of LDL	Macrophage degradation, μg/mg per 5 hr
Unincubated	0.98
Incubated (24 hr) with EC after	
No preincubation	6.42
Preincubation (5 hr, 37°C)	
Without *p*BPB	6.50
With 80 μM *p*BPB	0.50

^{125}I-LDL (200 μg) was incubated with or without 80 nmol of *p*BPB (added in 10 μl of warm ethanol) for 5 hr at 37°C in serum-free Ham's F-10 medium in a volume of 1 ml. At the end of the incubation, the volume was adjusted to 6 ml with F-10 medium, and LDL was reisolated by centrifugation at 40,000 rpm for 24 hr at ρ = 1.21, dialyzed against phosphate-buffered saline containing 0.01% EDTA, and then dialyzed against Ham's F-10 medium. The reisolated LDL was incubated with EC in serum-free F-10 medium at 100 μg/ml for 24 hr. After this incubation, macrophage degradation of LDL was measured. Results are averages of duplicate experiments.

Table 2. Failure of analogues of *p*BPB to affect either oxidation or biological modification of LDL by EC

Treatment of LDL	Thiobarbituric acid-reactive material, nmol/ml	Macrophage degradation, μg/mg per 5 hr
Unincubated	0.91	0.60
Incubated without EC	2.0	0.70
Incubated with EC		
Without additions	7.31	11.30
+ 100 μM Acetophenone	7.02	10.8
+ 100 μM *p*-Bromoacetophenone	6.73	10.8

LDL (200 μg of protein) was incubated in a volume of 2 ml with confluent EC or in no-cell control dishes for 24 hr. Acetophenone or *p*-bromoacetophenone was added in 10 μl of ethanol. Thiobarbituric acid-reactive materials and macrophage degradation of LDL were determined. Values shown are means of duplicate determination from a representative experiment.

Degradation of Apo B During EC Modification. As shown in Fig. 3, $NaDodSO_4$ gel electrophoresis of EC-LDL showed an almost complete loss of Coomassie blue staining in the B_{100} region (where B_{100} is the major form of apo B) (lane 3). The addition of either *p*BPB (lanes 4 and 5) or BHT (lane 6) to EC incubations prevented this loss of apo B. The very dense band with $M_r \approx 60{,}000$ appears at a comparable intensity in medium incubated with EC in the *absence* of LDL and is thus of cellular origin and, as seen in Fig. 3, unaffected by the inhibitors of EC modification. We considered the possibility that the modification might alter the affinity of the protein for Coomassie blue and repeated these studies with radioiodinated LDL. Autoradiograms of the gels again

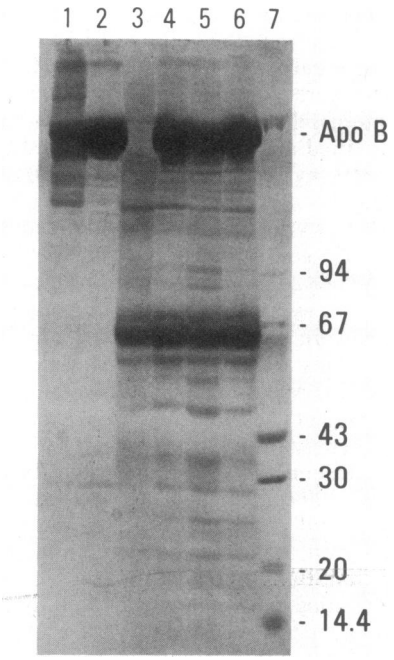

FIG. 3. $NaDodSO_4$ gradient gels of ^{125}I-LDL incubated with EC in the presence and absence of *p*BPB or BHT. Approximately 15 μg of ^{125}I-LDL protein for each preparation (30,000 cpm) was applied to the gel. Lane 1, LDL incubated in the absence of cells; lane 2, native LDL; lane 3, EC-LDL; lanes 4 and 5, EC-LDL modified in the presence of *p*BPB; lane 6, EC-LDL modified in the presence of BHT; lane 7, standard molecular weight markers shown as $M_r \times 10^{-3}$.

showed an almost complete disappearance of labeled protein from the B_{100} area and >95% of the ^{125}I applied was recovered in lower molecular weight bands. Also, samples were run at low concentrations and the gel was stained with silver. Again, the virtually complete loss of B_{100} was confirmed.

Cu^{2+}-Induced Modification of LDL in the Absence of Cells. Previously we have shown that incubation of LDL in F-10 medium in the presence of 5 μM Cu^{2+} for 24 hr can mimic most of the changes induced by incubating LDL overnight in the presence of EC, including the peroxidative changes, the changes in apo B, and the biologic modification (8). As shown in Table 3, *p*BPB at 30 μM completely inhibited the latter but inhibited peroxidation only to a limited extent. The hydrolysis of PtdCho was also completely prevented by *p*BPB (Fig. 4) as well as by BHT. These findings implied the presence of a phospholipase activity in LDL as it is isolated; this is explicitly shown by the data in Table 4. PtdCho incubated with native LDL released fatty acids from the 2-position at a significant rate. When the PtdCho substrate had been previously oxidized, the rate increased about 5-fold, in agreement with the previous findings of Sevanian *et al.* (15). Nonenzyme controls and boiled LDL controls showed essentially no activity.

DISCUSSION

*p*BPB is a potent, active site-directed inhibitor of snake venom and pancreatic phospholipase A_2 (16, 17). It also inhibits membrane-associated phospholipases in other systems (18, 19). Its ability in the present studies to completely inhibit the several changes in LDL induced by incubation with EC is most simply interpreted as a consequence of its ability to inhibit phospholipases. The possibility that it might act primarily as an antioxidant was considered, but closely related structural analogs lacking phospholipase inhibitory activity were without effect. Furthermore, at low *p*BPB concentrations there was at least a partial dissociation of its inhibitory effects on PtdCho degradation and biological modification, on the one hand, from its lesser effects on peroxidation, on the other. Finally, similar results were obtained when 1-bromooctan-2-one was used as inhibitor. Being aliphatic, this compound would not be expected to have antioxidant activity.

At least some, if not all, of the effects of *p*BPB can be attributed to its inhibition of phospholipase activity *associated with the LDL itself.* Pretreatment of LDL with *p*BPB prevented its subsequent EC modification. Furthermore, *p*BPB also inhibited the modification of LDL that occurs in the presence of higher concentrations of Cu^{2+} even in the absence of cells. These findings further support the interpretation that *p*BPB is effective because it inhibits phospholipase activity in the LDL, but other changes induced by *p*BPB could be of importance (e.g., reaction with amino acid residues in apo B). Although *p*BPB *can* inhibit EC modification by acting only on the LDL, that does not rule out additional effects on the cells. For example, inhibition of membrane phospholipases would be expected to decrease the generation of polyunsaturated free fatty acid substrate for prostaglandin-synthesizing enzymes, which, in turn, might be a source of free radicals involved in EC modification.

Which of the several changes in LDL structure account for the recognition of EC-LDL by the macrophage acetyl LDL receptor? The presence of l-PtdCho on the modified LDL is *not* essential. Incubation in the presence of a high concentration of albumin removed l-PtdCho from the EC-LDL but did not abolish its recognition by the acetyl LDL receptor (unpublished studies in collaboration with David S. Leake). Since the several chemical modifications that convert LDL to a form recognized by the acetyl LDL receptor all involve reaction with lysine residues of the apoprotein (4, 9, 10), the marked changes we observe in apo B both during EC modification and during exposure to Cu^{2+} in the absence of cells would appear to be the most likely basis for the change in recognition by the macrophage receptor. The striking changes in apo B associated with metal-catalyzed oxidation of LDL in the absence of cells have been reported previously by others (20, 21). Proteins exposed to extreme oxidative conditions lose significant numbers of lysine residues as well as other amino acid residues (22). Most recent-

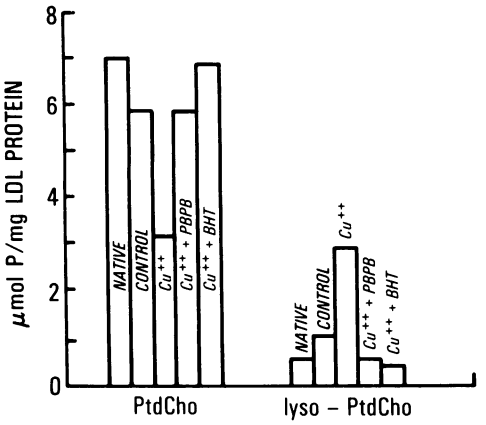

FIG. 4. Effect of *p*BPB on the hydrolysis of PtdCho during copper-induced modification of LDL. LDL (100 μg/ml) was incubated in 2 ml of Ham's F-10 medium in the presence of 5 μM Cu^{2+} and 100 μM *p*BPB or 40 μM BHT at 37°C for 16 hr. Lipids were extracted at the end of the incubation and were analyzed for PtdCho and l-PtdCho. Results are average values from a representative experiment.

Table 3. Effects of *p*BPB on copper-induced modification of LDL in the absence of cells

Treatment of LDL	Thiobarbituric acid-reactive material, nmol/ml	Macrophage degradation, μg/mg per 5 hr
Unincubated (100 μg/ml)	0.5	0.9
Incubated without additions	0.9	0.9
Incubated with 5 μM Cu^{2+}	4.6	9.8
+ 20 μM *p*BPB	4.5	5.0
+ 25 μM *p*BPB	4.4	3.6
+ 30 μM *p*BPB	3.9	0.9
+ 40 μM *p*BPB	3.5	0.8

LDL (200 μg of protein) was incubated with 5 μM copper sulfate in serum-free Ham's F-10 medium in a total volume of 2 ml at 37°C for 24 hr. *p*BPB was first added at indicated concentrations in 10 μl of warm ethanol and dried before the addition of other components. After incubation, thiobarbituric acid-reactive materials and macrophage degradation were measured. Values expressed are means of duplicates from a representative experiment.

Table 4. Phospholipase activity associated with native LDL

Substrate	"Enzyme"	Free fatty acids produced*, nmol/hr per mg of LDL protein
Unoxidized PtdCho	Buffer only	0.1 ± 0.01
	Native LDL	11.7 ± 0.15
Oxidized PtdCho	Native LDL	58.6 ± 2.6
	Boiled LDL	0.6 ± 0.07

*Includes oxidized and unoxidized fatty acids.

Oxidized or unoxidized 2-[^{14}C]linoleoyl-PtdCho was incubated with LDL. Products were separated by TLC and radioactivity associated with oxidized and unoxidized free fatty acids was determined. Enzyme activity is expressed as nmol of substrate hydrolyzed per hr/mg of LDL. Results are averages of duplicates from a representative experiment.

ly, studies by Stadtman and co-workers have shown that bacterial glutamine synthetase is "marked" for proteolytic degradation by a prior oxidative step and that this includes conversion of some lysine residues to the 5-keto derivative [(23); E. R. Stadtman and R. Levine, personal communication].

Can all of the observed changes and the inhibition of all of them *either* by antioxidants *or* by a phospholipase inhibitor be explained on a common basis? As a working hypothesis we suggest that oxidation and phospholipase activity are reciprocally reenforcing. Thus, the presence of an active phospholipase could release peroxidized free fatty acids and thus further the free radical-initiated peroxidation (*i*) by favoring propagation reactions, since the released fatty acids might move more freely within the structure of LDL or escape from it to interact with other LDL molecules or with EC, and (*ii*) by making it more likely that they can interact with apo B to modify its structure. Indeed, such free fatty acids may well be the component responsible for the cell toxicity of the lipid fraction from oxidized LDL demonstrated by Morel *et al.* (24). The ability of antioxidants to inhibit the overall process, including breakdown of PtdCho, could be explained on the basis that oxidation of fatty acids in PtdCho makes the latter a better substrate for phospholipase activity. Such an effect has indeed been reported in other systems (15, 25, 26) and is recognized as a possible basis for removal of toxic peroxides from structural phospholipids. Also, the possibility that oxidative changes somehow *activate* phospholipase must be considered.

Direct evidence for the role of EC modification in atherogenesis is still lacking. Smooth muscle cells can induce similar LDL modification (7), apparently by similar mechanisms (27), and macrophages may have the same potential. If these arterial cells play a role *in vivo*, the present findings imply that interventions to limit oxidative damage to LDL or to protect it from attack by phospholipase A_2 might prove antiatherogenic.

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