Dilinoleoylphosphatidylcholine protects human low density lipoproteins against oxidation

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Abstract

LDL oxidation may promote atherosclerosis. We found that polyenylphosphatidylcholine (PPC), a mixture of polyunsaturated phospholipids extracted from soybeans, has antioxidant effects in in vivo models of oxidative stress. To assess whether components of PPC affect the in vitro oxidizability of LDL, plasma from 15 healthy volunteers was incubated with 10 μM of either dilinoleoyl-, palmitoyl-linoleoyl-, linoleoyl-palmitoyl- or distearoyl-phosphatidylcholine as well as 10 μM α-tocopherol. LDL oxidation was initiated with 5 μM Cu²⁺ sulfate and monitored by conjugated diene production, or with 2,2’-azobis (2-amidinopropane) dihydrochloride, a free radical generator, and monitored by O₂ consumption. After addition of Cu²⁺, the lag phase (indicative of resistance of LDL to oxidation) was longer (140% of controls; P < 0.001) for LDL incubated with dilinoleoyl-, but not with the other phosphatidylcholine species. This effect was similar to that of 1 mM α-tocopherol (135%). After addition of 2,2’-azobis (2-amidinopropane) dihydrochloride, the inhibition time (also reflecting the antioxidant content of LDL) was prolonged (P < 0.001) for α-tocopherol (206%) and dilinoleoyl- (188%), but not for distearoyl-phosphatidylcholine. Thus, dilinoleoyl-phosphatidylcholine (the main component of PPC) protects against LDL oxidation, a possible mechanism for its reported anti-atherosclerosis effects. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Phospholipids; Anti-oxidants; Low-density lipoproteins; Atherosclerosis

1. Introduction

Growing evidence in human subjects indicates that oxidative modifications of plasma low density lipoproteins (LDL) enhance their atherogenic properties [1]. Peroxidation of LDL-lipids may increase atherogeneity by damaging the apoprotein B-100, thus reducing the normal catabolism through the regulated LDL receptor pathway and enhancing cholesterol uptake via an unregulated ‘scavenger’ pathway. The latter leads to cholesterol overloading of the macrophages in the arterial intima, engendering the ‘foam cells’, a key component of the atherosclerotic plaques [2].

Phosphatidylcholine (PC) extracted from soybeans retards the development of atherosclerosis in several animal models [3–8], but the mechanism remains speculative. More recently, a more purified polyenylphosphatidylcholine mixture (PPC), containing 94–96% phosphatidylcholines, 40–52% of which is dilinoleoyl phosphatidylcholine (DLPC), and 23–24% palmitoyl-linoleoyl phosphatidylcholine (PLPC), prevented the development of cirrhosis in alcohol-fed baboons [9].

It is generally believed that polyunsaturation of fatty acids favors their lipoperoxidation, because fatty acids with multiple double-bonds are much more vulnerable than saturated or monounsaturated ones to free radical attack. Indeed, a diet rich in polyunsaturated fatty acids increased the susceptibility of LDL to oxidation, compared with a diet high in monounsaturated and saturated fatty acids [10–14]. Surprisingly, however, recent evidence revealed striking ‘antioxidant’ effects of PPC in vivo [15] and in cultured hepatoma cell models of oxidative stress [16]. These effects were associated with normalization of peroxidation products, such as...
F2-isoprostane or 4-hydroxynonenal, and glutathione [15], raising the possibility that a component of this phospholipid mixture may act as an antioxidant. Moreover, chronic administration of 50% of calories as ethanol (corresponding to the average consumption of alcoholics) to baboons resulted in increased LDL-hydroperoxides, electronegativity of apo B100 and vulnerability of LDL to be oxidized (oxidizability), alterations which were markedly attenuated by supplementation of the alcohol-containing diet with PPC [17]. Therefore, the purpose of the present study was to determine whether any of the main phosphatidylcholine species of PPC possesses antioxidant properties and affects the in vitro oxidizability of human LDL.

2. Methods

2.1. Materials

1,2-Dilinoleoyl phosphatidylcholine (DLPC), 1,Palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) and 1,2,Distearyl phosphatidylcholine (DSPC) were purchased from Avanti Polar Lipids (Alabaster, AL). 1,Linoleoyl-2,palmitoyl phosphatidylcholine (LPPC) and 1,palmitoyl-2,linoleoyl phosphatidylcholine (PLPC) were obtained from Sigma (St Louis, MO) and α-tocopherol (vitamin E) from Aldrich (Milwaukee, WI).

2.2. Subjects

A total of 15 healthy volunteers (11 females and four males) participated in this study. Their age (mean ± S.E.M.) was 37.3 ± 7.5 years (range 21–65), their body weight was 59.5 ± 3.4 kg, and the body mass index (calculated by dividing the weight in kilograms by the square of height in meters) was 22.5 ± 0.99. Plasma total cholesterol was 4.9 ± 0.3 mmol/l (189.63 mg/dl), and plasma triacylglycerols were 1.18 ± 0.3 mmol/l (104.43 mg/dl). All subjects were in good physical condition at the time of study and, in particular, none of them was obese or hyperlipidemic. The study was approved by the institutional Human Research Committee and all subjects gave informed consent to participate in the study. Blood was collected in EDTA-containing tubes and centrifuged at 1200 × g for 20 min to separate the plasma.

2.3. Incorporation of phospholipids and vitamin E into plasma

The phospholipid species and the vitamin E were dissolved in absolute ethanol, dried under nitrogen, and incorporated into the plasma by incubation at 37°C for 3 h [17] to produce final concentrations of either 10 μM DLPC or 10 μM PLPC in plasma. Their effects were compared with those of 10 μM LPPC, 10 μM DSPC (a saturated phospholipid), and with either 10 μM or 1 mM α-tocopherol, a recognized antioxidant. In each subject, the plasma from which the control LDL was isolated, was incubated for 3 h at 37°C as for the plasma incubated with test phospholipids to allow for any consumption of endogenous LDL antioxidants during this treatment.

2.4. LDL isolation from plasma incubated with either phospholipids or vitamin E

LDL were isolated by a rapid technique [18]. Plasma (1 ml) was adjusted to a density of 1.21 g/ml with solid KBr in Quick-Seal polycrylomer tubes (Beckman Instruments, Palo Alto, CA) and spun at 260 000 × g for 45 min, without braking, in a vertical rotor. The locations of the lipoproteins were determined by staining plasma with Fat Red 7B (Helena Laboratories, Beaumont, TX). LDL, which appear as a distinct band, were removed through the side of the tube with a needle and syringe. The purity of the fraction was confirmed by its migration on agarose gel electrophoresis [19]. The isolated LDL were dialyzed overnight with two to three changes of phosphate buffer (pH 7.4), containing 150 mM NaCl (PBS), in the dark and at 4°C to remove KBr and EDTA. All buffers were deoxygenated by nitrogen bubbling before use, and the dialysis was performed in filled stoppered bottles at 4°C. LDL protein was measured by the method of Lowry [20].

2.5. LDL oxidation

Oxidation was performed within 48 h of LDL isolation. Oxidation kinetics of LDL were determined in vitro, using two methods described below.

2.5.1. Copper oxidation method

Treated and control LDL (100 μg protein/ml) were incubated with 5 μM Cu²⁺ sulfate in 2 ml of PBS buffer (pH 7.4) at 37°C for 8 h, by monitoring every 5 min the absorbance at 234 nm of conjugate dienes [21]. The plot of absorbance against time produces three phases: (a) a lag phase, (b) a propagation phase and (c) a decomposition phase. The lag time (which indicates the resistance of LDL to oxidation) was measured as the intercept between the baseline and the tangent of the absorbance curve during the propagation phase. The lag time in normal subjects has a reported range from 33 to 138 min [22], similar to the values found by us (Section 3). Although part of this variation may be due to ex vivo LDL oxidation (despite precautions), a valid comparison is against the subject’s own serum treated the same way as the samples exposed to the phospholipids, because the inter-assay coefficient of variation (CV = 4.36%) and the intra-subject one (CV = 16.6%) are comparatively much smaller [21].
The rate of diene production (which depends on the amount and type of oxidizable substrates) was calculated from the slope of the absorbance curve during the propagation phase. The maximum amount of conjugated dienes (at the interception between the propagation and decomposition phases) was calculated by using the extinction coefficient for conjugated dienes at 234 nm [21].

2.5.2. Thermal decomposition of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)

LDL (100 μg protein) was incubated in PBS to which AAPH was added, at a final concentration of 10 mM. AAPH is a water-soluble diazo compound that has the advantage of a constant yield of lipid hydroperoxides (whereas copper-induced oxidation depends on the initial release of free radicals). The LDL oxidation by AAPH was monitored by the oxygen consumption measured at 41°C with a Clark’s electrode [12]. Two phases with different rates of oxygen consumption were identified: (a) the inhibition and (b) the peroxidation phases with low and high rates of O₂ consumption, respectively. The length of the first phase (inhibition time, expressed in minutes) depends on the antioxidant content of LDL, reflecting the consumption of antioxidants as they react with free radicals. The rate of the steeper phase (peroxidation rate, expressed as nAtoms of O₂ consumed per minute) is an index of the susceptibility of LDL to oxidation once all antioxidants have been consumed.

![Graph](image)

Fig. 1. Effects of various phosphatidylcholines and α-tocopherol on the lag time during copper-mediated LDL oxidation, assessed by the conjugated diene method and expressed as per cent of the corresponding controls. Values are mean ± S.E.M. (n = 5–15). n-TOC, α-tocopherol; DLPC, 1,2, dilinoleoyl phosphatidylcholine; DSPC, 1,2, distearoyl phosphatidylcholine; LPPC, 1, linoleoyl-2, palmitoyl phosphatidylcholine; PLPC, 1, palmitoyl-2, linoleoyl phosphatidylcholine. ** P < 0.01, significantly different from control; *** P < 0.001, significantly different from control.

2.6. Statistics

All results are expressed as means ± S.E.M. The significance of the differences was assessed by parametric or non-parametric (Kruskal–Wallis on Ranks) one-way ANOVA, as appropriate, using SAS-PROC GLM procedure with SAS software (version 6.12; SAS Institute, Cary, NC). When effects from the ANOVA were significant, treatment means were compared by the Tukey’s multiple comparison test. A probability of < 0.05 was considered to be significant.

3. Results

During Cu²⁺-mediated oxidation, the lag times of control LDL were very variable, with a range from 38 to 172 min. Therefore, comparisons were made as percent of the corresponding controls (Fig. 1). The lag time was prolonged when LDL were incubated with DLPC (139.5 ± 5.7% of controls; P < 0.001), whereas it was not significantly affected by incubation with PLPC (120.1 ± 8.2%), the other main component of PPC (the soybean’s lecithin extract). The effect of DLPC was not reproduced by DSPC (76.7 ± 5.1% of controls), a phospholipid with a saturated fatty acid of the same carbon chain length as linoleate bound to the carbon 1 and 2 of the glycerol. Similarly, a phospholipid with an unsaturated fatty acid in position 1 and a saturated one in position 2, such as LPPC, did not reproduce the effect of DLPC (90.1 ± 9.0% of controls). The effect of 10 μM DLPC was similar to that of 1 mM α-tocopherol (135.4 ± 7.2% of controls), whereas an equivalent concentration (10 μM) of α-tocopherol showed no effect (98.0 ± 7.2% of controls).

During LDL oxidation by AAPH, the inhibition time was prolonged (P < 0.001) after incubation of LDL with either DLPC or 1 mM α-tocopherol, but not with DSPC (Table 1). When compared to the corresponding controls, the values with DLPC and α-tocopherol were 188 and 206% of the controls, respectively (Fig. 2).

By contrast, the incubation of LDL with the various phospholipids, as well as with α-tocopherol, did not change the rate of diene production or the maximum amount of dienes produced (Table 2). Similarly, the oxidation rate by the AAPH method also was not significantly affected by these agents (Table 1).

4. Discussion

Our results indicate that DLPC significantly protected human LDL against in vitro oxidation produced by either copper or AAPH. DLPC is the major component of PPC, a soybean derived phosphatidylcholine mixture. PPC has been reported to prevent atheroscle-
and O2 consumption. Whereas the release of free radi-
two types of measurements, namely diene conjugates
by copper and thermal decomposition of AAPH, and
two different sources of free radicals, namely oxidation
of lipoperoxidation. Similar results were obtained using
react with free radicals prior to reaching maximal rates
consumption of antioxidants present in LDL, as they
fested by a prolongation of the time needed for the
ported for
of LDL against lipoperoxidation. As previously re-
ted to position 2 [8].
which a single unsaturated fatty acid is bound, and only
to position 2 [8].

The incorporation of DLPC increased the resistance
of LDL against lipoperoxidation. As previously re-
ported for α-tocopherol [23,24], this resistance is mani-
fested by a prolongation of the time needed for the
consumption of antioxidants present in LDL, as they
react with free radicals prior to reaching maximal rates
of lipoperoxidation. Similar results were obtained using
two different sources of free radicals, namely oxidation
by copper and thermal decomposition of AAPH, and
two types of measurements, namely diene conjugates and O2 consumption. Whereas the release of free rad-
icals occurs predominantly at early times after copper
addition, their production remains constant with
AAPH [12], which probably accelerates the consump-
tion of antioxidants. Thus, despite their quantitative
differences, both the lag and the inhibition times (ob-
served with these methods) depend on the antioxidant
content of the LDL. Once all antioxidants were con-
sumed, neither the rate of LDL oxidation nor the
amount of dienes produced were changed by incubation
with DLPC, other phosphatidylcholines or α-toco-
phol. This indicates that the amount of substrate
available for oxidation was not significantly altered by
the additions.

It appears paradoxical that DLPC displays antioxi-
dant properties despite being rich in unsaturated fatty
acids, main substrates for lipoperoxidation. However,
the possibility that this phospholipid could act as an
antioxidant was suggested because of the results ob-
tained after in vivo administration of a DLPC-rich
mixture (PPC) to baboons fed alcohol [15] and to rats
with γtocopherol [26,27] and CCl4 [25] as well as after addition of the component phospholipids to cultured hepatoma cells
undergoing an oxidative stress [16]. The possibility that
the decrease in lipoperoxidation indexes could have
been secondary to improvement of other cell functions
is excluded in this in vitro study, which reveals a direct
antioxidant effect of DLPC. The exact mechanism by
which this phospholipid acts as an antioxidant is un-
known, but appears to be specific for DLPC, since its
antioxidant property was not shared by phospholipids
containing either saturated fatty acids (of the same
chain length as linoleate) in both positions 1 and 2 or a
single linoleate bound to either position 1 or 2 of the
glycerol.

The antioxidant capacity of DLPC for LDL appears
to be much greater than that of α-tocopherol: the
prolongation of either the lag or the inhibition phases
by 10 μM DLPC in plasma was similar to that pro-
duced by 1mM α-tocopherol, whereas 10 μM α-toco-
pherol had no detectable effect. This difference is not
likely to be due to a more efficient uptake of DLPC
than α-tocopherol into LDL, because the uptake of
α-tocopherol [26,27] was greater than that of DLPC
[28], under conditions similar to ours. Incubation of
plasma with 1 mM α-tocopherol at 37°C for 3 h
increased LDL-tocopherol from 5 to 20 μg (12–46
nmol) per mg of LDL-protein [26,27]. On the other
hand, in the vitro incorporation of DLPC into LDL

| Table 1 |

<table>
<thead>
<tr>
<th>Effect of phosphatidylcholines and α-tocopherol during 2,2'-azobis (2-amidinopropane) dihydrochloride-mediated LDL oxidation measured by the oxygen consumption method*</th>
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<tbody>
<tr>
<td>Control, n = 9</td>
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<tr>
<td>Inhibition time (min)</td>
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<td>Oxidation rate (nAtoms/min per mg protein)</td>
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<td><strong>P</strong></td>
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* Values are mean ± S.E.M. α-TOC, α-tocopherol; DLPC, 1,2,dlinoleoyl phosphatidylcholine; DSPC, 1,2,distearoyl phosphatidylcholine.

** Overall P-value from one-way ANOVA; NS, not significant (P ≥ 0.05).

a Significantly different from control.

Fig. 2. Comparison of the effects of polyunsaturated (dlinoleoyl-) and saturated (distearoyl-) phosphatidylcholines and α-tocopherol on the inhibition time during 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-mediated LDL oxidation, assessed by oxygen consumption and expressed as percent of the corresponding controls. Values are mean ± S.E.M. (n = 5–9). α-TOC, α-tocopherol; DLPC, 1,2,dlinoleoyl phosphatidylcholine; DSPC, 1,2,distearoyl phosphatidylcholine. *** P < 0.001, significantly different from control.
<table>
<thead>
<tr>
<th>Rate of diene production (nmol/min)</th>
<th>Control, $n = 15$</th>
<th>+ DLPC (10 μM), $n = 15$</th>
<th>+ PLPC (10 μM), $n = 15$</th>
<th>+ LPPC (10 μM), $n = 7$</th>
<th>+ DSPC (10 μM), $n = 5$</th>
<th>+ α-TOC (10 μM), $n = 6$</th>
<th>+ α-TOC (1 mM), $n = 7$</th>
<th>$P^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximal amount of dienes (nmol/mg protein)</td>
<td>0.63 ± 0.10</td>
<td>0.60 ± 0.10</td>
<td>0.51 ± 0.08</td>
<td>0.60 ± 0.10</td>
<td>0.57 ± 0.06</td>
<td>0.50 ± 0.09</td>
<td>0.66 ± 0.09</td>
<td>NS</td>
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<tr>
<td></td>
<td>340 ± 44</td>
<td>338 ± 49</td>
<td>336 ± 48</td>
<td>332 ± 62</td>
<td>364 ± 55</td>
<td>387 ± 61</td>
<td>320 ± 99</td>
<td>NS</td>
</tr>
</tbody>
</table>

$^a$ Values are mean ± S.E.M. α-TOC, α-tocopherol; DLPC, 1,2, dilinoleoyl phosphatidylcholine; DSPC, 1,2, distearoyl phosphatidylcholine; LPPC, 1, linoleoyl-2, palmitoyl phosphatidylcholine; PLPC, 1, palmitoyl-2, linoleoyl phosphatidylcholine.

$^b$ Overall $P$-value from one-way ANOVA; NS, not significant ($P \geq 0.05$).
was studied by Zierenberg et al. [28], who found that 5–14% of 1 mg labeled DLPC was incorporated into human LDL. This corresponds to 1–2.8 μg (2.3–3.6 nmol) per mg of LDL-protein. In addition, ~50% of the DLPC incorporation occurred in HDL [28]. It has been suggested that the protective role of HDL on atherogenesis could be mediated, at least in part, by preventing the generation of oxidized LDL [29]. Intravenous administration of doubly labeled DLPC has shown that this lecithin species is incorporated in toto and unaltered into the cell membranes [29]. The in vitro incorporation of DLPC into LDL was similar to that after intravenous administration of the dose [28].

A DLPC-containing mixture extracted from soybeans retards the development of atherosclerosis in hypercholesterolemic rabbits [2], baboons [3,4], Japanese quails [5], minipigs and rats [6]. Supplementation with α-tocopherol provides some protection against coronary artery disease [30–32]. By analogy, the findings of the present study suggest that the preventive effect of DLPC on atherosclerosis is mediated, at least in part, by its protective effect on LDL oxidation. Thus, because of its antioxidant capacity and its apparent lack of toxicity [8], DLPC is thus a good candidate to be used for the prevention of atherosclerosis.

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