Oxidation of LDL in baboons is increased by alcohol and attenuated by polyenylphosphatidylcholine

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Abstract **Alcohol taken in moderation may prevent atherosclerosis, whereas heavy drinking has the opposite effect, in part by promoting oxidation of low density lipoproteins (LDL), a pathogenetic factor in atherogenesis. We assess here: *1*) whether similar alterations can be reproduced in baboons fed 50% of energy as ethanol (the average intake of alcoholics) for 7–8 years, and *2*) whether such alterations are affected by supplementation with polyenylphosphatidylcholine (PPC), a mixture of polyunsaturated phosphatidylcholines, shown to prevent alcoholic fatty liver, fibrosis, and cirrhosis. Ten animals were given the ethanol-containing diet and ten were pair-fed isocaloric control diets. In half of the pairs, the diets were supplemented with 2.8 g of polyenylphosphatidylcholine/1000 kcal. Alcohol feeding increased LDL-lipoperoxides and made LDL-proteins more negatively charged, changes that were attenuated or prevented by PPC. The oxidizability of LDL was determined in vitro by the formation of conjugated dienes after oxidation with copper. Alcohol shortened the lag time (which measures LDL antioxidant capacity); this effect was normalized by PPC supplementation. By contrast, PPC produced no changes in the controls. Thus polyenylphosphatidylcholine, by markedly attenuating the ethanol-induced increase in LDL oxidation, opposes one of the effects whereby alcohol promotes atherosclerosis.**—Navder, K. P., E. Baraona, M. A. Leo, and C. S. Lieber. **Oxidation of LDL in baboons is increased by alcohol and attenuated by polyenylphosphatidylcholine.** ***J. Lipid Res.*** **1999.** 40: **983–987.**

Supplementary key words alcohol • atherosclerosis • LDL oxidation • polyenylphosphatidylcholine • dilinoleoyl-phosphatidylcholine • baboons

Alcohol consumption exerts a dual type of effect on the development of atherosclerosis and coronary heart disease: it is considered to be protective in moderate amounts, but aggravates these conditions in heavy drinkers, resulting in a J- or U-shaped dose–effect curve (1, 2).

The protective effect has been linked to the increase in high density lipoprotein (HDL)-cholesterol. This increase results not only from an enhancement of the reversed cholesterol transport from vessels to the liver, secretion into the bile, and fecal excretion (3–7), but it also reflects a defect in the transfer of cholesteryl ester from HDL to the VLDL-LDL pathway (6, 8), an important step in the disposition of cholesterol via hepatic apoB-E receptor in primates.

While a positive correlation between coronary artery disease and serum levels of low density lipoproteins (LDL) is well recognized (9), it has been suggested that oxidatively modified LDL rather than native unmodified LDL are responsible for atherogenesis (10, 11). Ethanol increases lipid peroxidation in the liver (12–14). As ethanol promotes oxidative stress and changes the concentrations of antioxidants in the liver (15), it is possible that secreted lipoproteins may undergo oxidative modification during secretion or may be depleted of antioxidants. In fact, Lin et al. (16) demonstrated that the oxidation of LDL was increased in alcoholics. In the present study, we assessed whether a similar alteration occurs under nutritionally controlled conditions in baboons fed 50% of energy as ethanol (an amount equivalent to the average consumption of alcoholics) and whether these alterations can be affected by polyenylphosphatidylcholine (PPC), a polyunsaturated lecithin extracted from soybeans and shown to prevent the development of alcoholic fatty liver (17) as well as fibrosis and cirrhosis (18) in experimental animals. Moreover, phosphatidylcholine extracted from soybeans was found to retard the development of atherosclerosis in several animal models (19–22), but the mechanism of this effect remains speculative.

MATERIALS AND METHODS

Diets and other materials

The solid components of the liquid diets used in this study were purchased from Dyets, Inc. (Bethlehem, PA). Their detailed

Abbreviations: LDL, low density lipoproteins; PPC, polyenylphosphatidylcholine; DLPC, dilinoleoyl-phosphatidylcholine; PLPC, palmitoyl-linoleoyl-phosphatidylcholine.

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composition has been previously reported (23). Their energy distribution was 18% as protein, 21% as fat, 11% as carbohydrate, and 50% either as ethanol or as additional carbohydrate. The diets contained 30 IU/liter of vitamin E, which is at least twice the level recommended for baboons (24). PPC was kindly provided by Rhone-Poulenc Rorer GmbH (Köln, Germany). Its detailed composition has been determined (18): it contains 94–96% phosphatidylcholines, with the two major species being dilinoleoylphosphatidylcholine (DLPC) (40–52%) and 1,palmitoyl-2,linoleoylphosphatidylcholine (PLPC) (23–24%). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Animal procedures

Twenty *Papio hamadryas* (10–34.5 kg; 8 males and 12 females) were studied in compliance with the institution's guidelines for animal research. All procedures followed the National Research Council's criteria for animal care. For an average of 7.3 ± 0.4 years, 10 animals consumed ad libitum the ethanol-containing diet and 10 were pair-fed the isocaloric control diet. In half of the pairs, the diets were supplemented with 2.8 g of PPC/1000 kilocalories, every other day. No oxidative products (4-hydroxy nonenal) were detected by HPLC in either PPC or PPC-containing diet.

Six hours after alcohol withdrawal, blood was collected in EDTA-containing tubes under ketamine anesthesia (10–20 mg/kg) and a needle liver biopsy was performed.

LDL isolation

Plasma was separated by centrifugation of blood at 1200 *g* for 20 min. LDL were isolated by a rapid technique (25): Plasma (1 ml), pre-stained with Fat Red 7B (Helena Laboratories, Beaumont, TX), was adjusted to a density of 1.21 g/ml with solid KBr in Quick-Seal polyallomer tubes (Beckman Instruments Inc, Palo Alto, CA) and spun at 260,000 *g* for 45 min, without braking, in a vertical rotor (Beckman VTi 65 rotor). LDL, which appear as a distinct amber band, were removed through the side of the tube with needle and syringe. The purity of the fraction was confirmed by its migration on agarose gel electrophoresis (26). The isolated LDL were dialyzed overnight with 2–3 changes of phosphate buffer (pH 7.4), containing 150 mm NaCl (PBS), at 4°C in the dark, to remove KBr and EDTA. All buffers were deoxygenated by nitrogen bubbling before use, and the dialysis was performed in filled stoppered bottles. LDL protein was measured by the method of Lowry et al. (27).

LDL lipid peroxides

Lipid peroxides in LDL samples were processed in the dark and quantified colorimetrically at the absorbance of methylene blue (675 nm) against the cumene hydroperoxide standard provided with the assay kit purchased from Kamiya Biomedical Co. (Seattle, WA). In principle, hydroperoxides were reduced to lipid alcohols in the presence of hemoglobin, and this reaction was coupled to the oxidation of 10-N-methylcarbamoyl-3,7-dimethylamino-10 H-phenothiazine, which splits methylene blue in an equal molar reaction.

LDL electromobility

Lipoperoxidation is associated with changes of apolipoprotein B-100 due, at least in part, to the formation of protein adducts with aldehyde products of lipoperoxidation, especially at the ε-amino groups of lysine, thereby changing LDL electrophoretic mobility. The migration of LDL was assessed on agarose gel electrophoresis (26), using the Titan Gel Lipoprotein Electrophoresis System (Helena Laboratories, Beaumont, TX). LDL samples (5 μg of protein) were loaded on each lane and run for 45 min under a constant voltage (60 V) at room temperature. The gel was then stained with a methanol solution of Fat Red 7B to develop the lipoprotein bands. The mobility of LDL was determined by measuring distance of migration from point of application (origin) toward the anode.

LDL oxidation by copper and formation of diene conjugates

The oxidizability of LDL was determined in vitro by measuring the formation of diene conjugates after oxidation with copper (28). Within 24–30 h of isolation, LDL (100 μg protein/ml) were incubated with 5 μm Cu^{2+} sulfate in 2 ml of PBS buffer (pH 7.4) at 37°C for 8 h, by monitoring the absorbance at 234 nm of diene conjugates every 5 min. The plot of absorbance against time has 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 rate of oxidation (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 diene conjugates produced by the oxidation procedure was calculated using the maximal absorbance at 234 nm at the interception between the propagation and decomposition phases. The dienes were calculated by using the extinction coefficient for diene conjugates at 234 nm (29500 $L \cdot mol^{-1} \cdot cm^{-1}$).

Statistics

All results are expressed by their means ± SEM. The significance of the differences was assessed by two-way ANOVA for the effects of alcohol, PPC and their interaction, using SAS (29). When effects from ANOVA were significant, treatment means were compared by the Tukey's multiple comparison tests. A probability level of <0.05 was considered to be significant.

RESULTS

Of the 5 baboons given the alcohol diet alone, all had fatty liver, 2 had perivenular fibrosis, 2 had septal fibrosis, and 1 had developed cirrhosis. All the 5 baboons given the diet with both alcohol and PPC also had fatty liver, 3 of them had perivenular fibrosis, but none had developed septal fibrosis or cirrhosis. The control baboons, with or without PPC, had normal liver histology.

The ethanol consumption was not affected by the supplementation with PPC (4.42 ± 0.35 g/kg per day with PPC versus 4.73 ± 0.32, without PPC; nonsignificant). In the alcohol-fed animals, the changes reported below did not appear to correlate with either the time on alcohol or the severity of liver injury.

Lipid peroxides in LDL

Alcohol feeding resulted in a striking increase of lipid peroxides in LDL (**Fig. 1**). The effects of alcohol, PPC, and their interaction were statistically significant ($P < 0.01$) by 2-way ANOVA. Compared to their pair-fed controls, both groups of alcohol-fed baboons exhibited marked increases in lipid peroxides in LDL. But the supplementation of the alcohol-containing diet with PPC significantly ($P < 0.01$) lowered (by approximately half) the ethanol-induced increases in LDL lipoperoxides (from 349.2 ± 9.8 to 180.8 ± 16.9 nmol/mg protein with PPC), whereas PPC did not alter these values in the pair-fed controls.

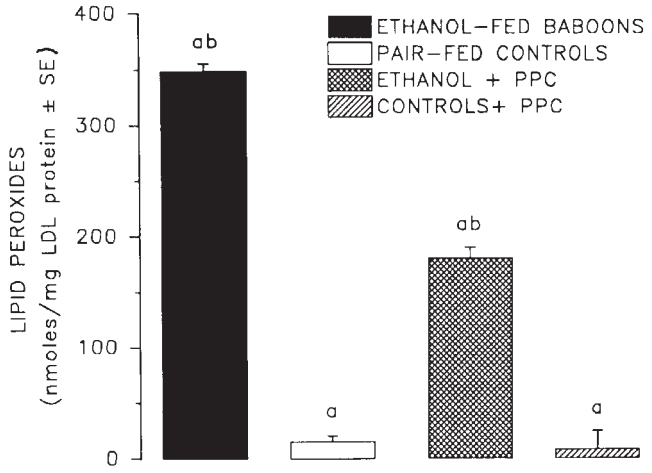

Fig. 1. Effects of ethanol and/or PPC feeding on LDL-lipid peroxides in the baboon. Data are mean ± SEM. Significant differences ($P < 0.01$), a: between alcohol-fed baboons and pair-fed controls, b: between alcohol-fed baboons given diets with or without PPC.

Electromobility of LDL

The changes in LDL-lipids were associated with parallel changes in LDL protein, which resulted in a change in electrophoretic mobility. LDL from the alcohol-fed baboons were more negatively charged and migrated faster toward the anode compared to those of their pair-fed controls (**Fig. 2**). Without PPC, the mean mobility of LDL from alcohol-fed baboons was significantly higher than in the controls (12.0 ± 1.4 mm vs. 7.6 ± 0.7, in the controls; $P < 0.05$). Supplementation with PPC prevented the increase in mobility induced by ethanol (8.0 ± 1.1 mm vs. 7.6 ± 1.0 in the controls; nonsignificant). PPC did not significantly affect the electromobility of LDL in the controls (7.6 ± 1.0 mm with PPC vs. 7.6 ± 0.7, without PPC; nonsignificant).

In vitro oxidizability of LDL

The main alteration in the formation of diene conjugates produced by alcohol feeding (after oxidation of LDL with copper) was a marked shortening of the lag time preceding the propagation phase. The latter phase proceeded with a higher rate of oxidation and resulted in increased amount of dienes in the alcohol-fed animals (**Table 1**). Supplementation of diets with PPC normalized the ethanol-induced decrease in lag time, whereas this supplementation produced no changes in the controls. The ethanol-induced increases in the rate of oxidation and in the maximal amount of dienes produced were attenuated by the supplementation with PPC.

No gender differences were noted in the effects of either alcohol or PPC, but the statistical power was too small for a gender comparison.

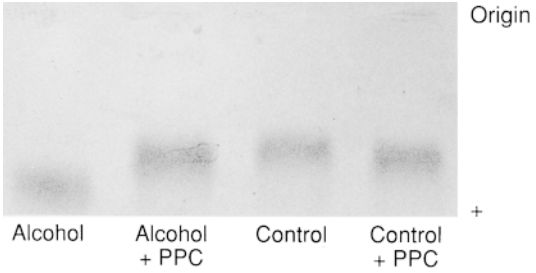

Fig. 2. Effect of alcohol and/or PPC feeding on the mobility of baboon LDL on agarose gel electrophoresis (amplification × 3). Alcohol feeding resulted in increased mobility of LDL toward the cathode. Supplementation with PPC prevented this effect. By contrast, PPC had no effect in the controls.

DISCUSSION

Our results in baboons indicate that supplementation of the diet with PPC attenuates the effects of ethanol consumption on LDL lipoperoxides, electromobility, and in vitro oxidizability by copper.

Ethanol consumption has been shown to produce lipoperoxidation in the liver of baboons (13) and of alcoholics (14). The increase in lipoperoxides in LDL from alcohol-fed baboons (Fig. 1) is consistent with similar changes reported by Lin et al. (16) in alcoholics. As observed in alcoholics, the peroxidation of the lipid moiety of LDL was associated with alterations of the protein moiety, resulting in a change of electrophoretic mobility. When subjected to electrophoresis on agarose gel, the LDL of alcohol-fed baboons migrated faster toward the anode than LDL from the controls (Fig. 2), indicating a decrease in positive charge. This has been attributed to the formation of adducts between the ε-amino group of lysine in apolipoprotein B and aldehydes, either those derived from lipoperoxidation (such as malondialdehyde and 4-hydroxynonenal) (16) or from the oxidation of ethanol (such as acetaldehyde) (30). Supplementation of the diets with PPC strikingly prevented or attenuated the ethanol-induced changes in LDL, whereas it produced no significant changes in the controls.

The degree of lipoperoxidation also depends upon the presence of antioxidants in LDL. The level of α-tocopherol (vitamin E) was found to be decreased in the liver (31) and in the LDL of alcoholics (16). To assess the capacity of LDL to resist oxidation, we measured the time taken by copper-induced oxidation to result in accelerated production of diene conjugates (32). This lag time is thought to be due to the chain-braking effects of antioxidants (such as α-tocopherol). Only when antioxidants are depleted does the lipid peroxidation cascade proceed to attack the remainder of the fatty acid chain with rapid production of diene conjugates. Alcohol feeding markedly decreased this lag time, indicating that the antioxidant capacity of LDL from alcohol-fed baboons was less than that of controls (Table 1). As a consequence, their LDL were more vulnerable to oxidation. This effect on the lag time was associated with an accelerated rate of diene production, resulting in a greater amount of diene conjugates formed. These differences are probably due to the higher unsaturated/saturated fatty acid ratio reported

TABLE 1. Effects of ethanol and/or polyenylphosphatidylcholine (PPC) on the susceptibility of LDL to oxidation as determined by production of diene conjugates

	Without PPC		With PPC		Statistical Significance[a,b] (P)		
	Alcohol	Control	Alcohol	Control	A	P	I
Lag time (min)	56 ± 12[c,d]	164 ± 12	124 ± 2[d]	142 ± 47	<0.05	ns	ns
Rate of oxidation (nmols/min)	0.25 ± 0.05[c]	0.11 ± 0.01	0.19 ± 0.02[c]	0.12 ± 0.02	<0.01	ns	ns
Maximal dienes (nmols/mg prot)	341 ± 13[c]	201 ± 11	296 ± 8[c]	195 ± 8	<0.01	ns	ns

Values are given as means ± SEM; n = 5 baboons/group.
[a] Overall *P* values from 2-way ANOVA; ns, not significant, $P \geq 0.05$.
[b] A, alcohol; P, PPC; I, interaction.
[c] Significant differences between alcohol-fed baboons and their pair-fed controls.
[d] Significant differences between alcohol-fed baboons given diets with and without PPC.

in alcohol-fed animals (33). PPC almost completely corrected the lag time, but only partially attenuated the changes in the rate of oxidation and in maximal diene conjugate production (Table 1).

It seems paradoxical that PPC (a soybean-derived phosphatidylcholine mixture rich in polyunsaturated fatty acids) did not exacerbate but, in fact, strikingly attenuated ethanol-induced lipid peroxidation. Indeed, it is well established that diets rich in polyunsaturated fatty acids (mainly as triglycerides) increase the susceptibility of LDL to be oxidized, compared with a diet high in monounsaturated and saturated fatty acids (34–36). However, the administration of the unsaturated fatty acids bound to soybean-derived phosphatidylcholines decreased indexes of ethanol-induced lipoperoxidation in the liver, such as 4-hydroxynonenal and F_2-isoprostanes (37). The main component of PPC, namely dilinoleoyl-phosphatidylcholine (DLPC), differs chemically from the structure of mammalian phospholipids by the presence of unsaturated fatty acid not only in the position 2, but in both positions 1 and 2 of the glycerol backbone. Physiologically, PPC-derived DLPC is characterized by a high bioavailability (38) and strikingly unaltered incorporation into cell membranes (39), which may be responsible for its antioxidant properties. In a recent study, we compared the in vitro effects of several soybean-derived phospholipids on the oxidizability of human LDL. DLPC, but not other components of soybean's polyenylphosphatidylcholine, was found to protect against LDL oxidation, which could be a possible mechanism for the reported anti-atherosclerotic effects (19–22). The antioxidant effect was comparable to that of a 10-fold higher concentration of α-tocopherol (vitamin E), which has been used with encouraging results in the prevention of atherosclerosis and coronary heart disease (40, 41).

In addition to its antioxidant properties, PPC may contribute to the prevention of coronary heart disease in moderate drinkers by maintaining the ethanol-induced increase in HDL and increasing the HDL/LDL cholesterol ratio even further (42).

The beneficial effects of PPC on alcoholic liver injury (18), if confirmed by ongoing multicenter trials, could result in substantial and chronic use of this phospholipid among alcoholics. It is therefore important to note that, despite being high in polyunsaturated fatty acids, this phospholipid does not favor LDL oxidation, but actually decreases it.

This work was supported by National Institutes of Health grants AA 05934, AA 11115, and AA 11160, Department of Veterans Affairs, and Kingsbridge Research Foundation.

Manuscript received 30 November 1998 and in revised form 8 February 1999.

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