

Lipoprotein-X reduces LDL atherogenicity in primary biliary cirrhosis by preventing LDL oxidation

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Abstract Hypercholesterolemic human LDL contains oxidized subfractions that have atherogenic properties. Paradoxically, atherosclerosis incidence is low in patients with primary biliary cirrhosis (PBC), a disease characterized by marked increases in plasma LDL, including the LDL subfraction lipoprotein-X (Lp-X). To investigate the mechanisms underlying this paradox, we first examined the propensity to oxidation of unfractionated LDL isolated from PBC patients. After prolonged incubation with copper, PBC-LDL failed to increase the oxidation index or electrophoretic mobility noted in control LDL. An admixture of PBC-LDL or Lp-X with control LDL prevented oxidation of the latter in a dose-dependent manner. PBC-LDL was also noncompetitive against copper-oxidized LDL (oxLDL) for binding with a murine monoclonal anti-oxLDL antibody in a competitive ELISA. OxLDL exerts its proapoptotic and antiangiogenic effects in part by inhibiting fibroblast growth factor 2 (FGF2) expression. Preincubation of oxLDL with PBC-LDL, but not control LDL, attenuated the inhibitory effects of oxLDL on FGF2 expression in cultured bovine aortic endothelial cells (ECs). The antioxidant and prosurvival properties of PBC-LDL diminished after the patients underwent orthotopic liver transplantation. These results suggest that Lp-X reduces LDL atherogenicity by preventing LDL oxidation to protect EC integrity in the presence of hypercholesterolemia. They also suggest that altering LDL composition may be as important as reducing LDL concentration in preventing or treating atherosclerosis.—Chang, P-Y., S-C. Lu, T-C. Su, S-F. Chou, W-H. Huang, J. D. Morrisett, C-H. Chen, C-S. Liao, and Y-T. Lee. **Lipoprotein-X reduces LDL atherogenicity in primary biliary cirrhosis by preventing LDL oxidation.** *J. Lipid Res.* 2004. 45: 2116–2122.

Supplementary key words hypercholesterolemia • low density lipoprotein oxidation • fibroblast growth factor 2 • endothelium

Hypercholesterolemia increases atherosclerosis incidence in the general population but not in patients with primary biliary cirrhosis (PBC), a cholestatic liver disease often as-

sociated with marked increases in plasma LDL cholesterol (LDL-C) (1–3). Despite the highly increased cholesterol levels, prospective observation for a median of 7.4 years of 312 patients with PBC of various stages found no increased incidence of atherosclerotic death compared with age- and sex-matched controls (4). Factors that may account for this apparent paradox are not clearly defined. Lipoprotein-X (Lp-X), an LDL rich in phospholipid and non-esterified cholesterol but poor in cholesteryl ester, triglyceride, and protein, is characteristic of advanced PBC (3). In all three isoforms, Lp-X₁, Lp-X₂, and Lp-X₃, the particles are rich in phospholipids (65–67%) and cholesterol (23–27%) but poor in cholesteryl esters (0.5–2%), triglycerides (2–3%), and protein (3–7%), giving them a hydrated density similar to that of LDL (5). The lack of association between high LDL-C and atherosclerosis in PBC raises the question of the atherogenic properties of PBC-LDL and prompted us to investigate the physiological relevance of Lp-X in atherogenesis.

The bioactivities of oxidized products of LDL may account for LDL atherogenicity (6). If PBC-LDL is resistant to oxidation, a lesser amount of such oxidized metabolites will be accumulated. Therefore, we first assessed the susceptibility of PBC-LDL to oxidation. Because PBC-LDL may contain a variable percentage of Lp-X, we also tested whether Lp-X renders LDL resistant to oxidants. Fibroblast growth factor 2 (FGF2), by functioning as an autocrine and paracrine agent, regulates the proliferation and apoptosis of vascular endothelial cells (ECs) by activating the phosphatidylinositol 3-kinase-protein kinase Akt pathway (7, 8). Both copper-oxidized LDL (oxLDL) and hypercholesterolemic electronegative LDL inhibit proliferation and

Abbreviations: BAEC, bovine aortic endothelial cell; EC, endothelial cell; FGF2, fibroblast growth factor 2; LDL-C, low density lipoprotein cholesterol; Lp-X, lipoprotein-X; oxLDL, copper-oxidized low density lipoprotein; PBC, primary biliary cirrhosis; TBARS, thiobarbituric acid-reactive substances.

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induce apoptosis in ECs in part by downregulating expression of the FGF2 gene (9–11). Whether PBC-LDL regulates FGF2-mediated survival mechanisms depends on its effect on FGF2 expression. This important issue was also addressed in this study.

MATERIALS AND METHODS

Materials

EDTA was from Becton-Dickinson (Rutherford, NJ). The Superose 6HR 10/30 column was from Pharmacia LKB (Uppsala, Sweden). Enzymatic kits for cholesterol and triglyceride analyses were from E. Merck (Darmstadt, Germany). The Beckman Paragon system was from Beckman (Palo Alto, CA). DMEM was from Invitrogen (Carlsbad, CA). Nonidet P-40 was from Sigma (St. Louis, MO).

LDL, Lp-X, and oxLDL preparation

LDL ($d = 1.019\text{--}1.063\text{ g/ml}$) was isolated by sequential ultracentrifugation from the plasma of six PBC patients after fasting (Table 1). To isolate Lp-X, PBC-LDL and LDL obtained from healthy, normolipidemic subjects were subjected to gel filtration chromatography using a Superose 6HR 10/30 column at 0.25 ml/min and collected in 0.5 ml fractions (12). OxLDL was prepared by incubating control LDL with $10\text{ }\mu\text{M}$ CuSO_4 for 24 h as previously described (13). Protein in LDL preparations was estimated by the Lowry method (14), and thiobarbituric acid-reactive substances (TBARS) were assayed as a measure of oxidative lipid modification (13). Cholesterol and triglyceride concentrations in plasma and LDL preparations were determined enzymatically as previously described (15).

Oxidation and oxidizability analyses

The oxidizability of LDL preparations was determined by their electrophoretic mobility after being exposed to $10\text{ }\mu\text{M}$ CuSO_4 at 37°C for 1–18 h on agarose gels using the Beckman Paragon system (16). A competitive ELISA was established using an oxLDL-specific monoclonal antibody, mAb-4E6, a generous gift from Professor Paul Holvoet at the Center for Experimental Surgery and Anesthesiology, Leuven, Belgium (17). In brief, oxLDL ($1\text{ }\mu\text{g/ml}$, $100\text{ }\mu\text{l/well}$) was used as the plated antigen. mAb-4E6 was added in the presence or absence of competing ligands: oxLDL, PBC-LDL, and control LDL. Samples were incubated for 2 h at room temperature. After washing, the wells were incubated for 1 h with horseradish peroxidase-conjugated rabbit IgG raised against mouse immunoglobulins and washed again. The peroxidase reaction was performed as previously described (17), and the absorbance was read at 492 nm .

Cell culture

Primary cultures of bovine aortic endothelial cells (BAECs) were derived and purified as previously described (18). Cells at 8–12 passages, maintained in DMEM supplemented with 10% FBS and antibiotics ($100\text{ }\mu\text{g/ml}$ streptomycin, 100 IU/ml penicillin, and $0.25\text{ }\mu\text{g/ml}$ amphotericin B), were used. Cell cultures grown to subconfluence were washed three times with serum-free medium and maintained under serum-free conditions for 6 h before being treated with PBS (lipoprotein-free control), control LDL ($50\text{ }\mu\text{g/ml}$), oxLDL ($50\text{ }\mu\text{g/ml}$), or PBC-LDL ($50\text{ }\mu\text{g/ml}$) for 24 h. For the study of DNA synthesis and intracellular FGF2 protein, 1×10^6 cells were seeded in each well of 12-well Corning cell culture plates.

Determination of intracellular FGF2 protein and mRNA levels

Intracellular FGF2 concentrations in cell lysates prepared with Nonidet P-40 were measured by ELISA using a Quantikine kit. As previously described (18), FGF2 concentrations were estimated at 450 nm spectrophotometrically using standard curves. FGF2 mRNA levels were determined by RT-PCR; $1\text{ }\mu\text{g}$ of the extracted total RNA was reverse transcribed in $10\text{ }\mu\text{l}$ of reaction mixture containing 2.5 units of Moloney murine leukemia virus reverse transcriptase for 45 min at 42°C . RT-PCR was performed according to procedures described previously (10). The FGF2 primers were 5'-GGAGTGTGTGCTAACCGTTACCTGGCTATG-3' (upstream) and 5'-TCAGCTCTTAGCAGACATTGGAAGAAAAAG-3' (downstream). As an internal control, β -actin was used with the following primers: 5'-AACCGCGAGAAGATGACCCAGATCATGTTT-3' (upstream) and 5'-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3' (downstream). A fraction of each PCR product ($10\text{ }\mu\text{l}$) was electrophoresed on a 2% agarose gel, and DNA bands stained with ethidium bromide were visualized by UV transillumination.

Statistical analysis

The significance of the differences between group means was assessed by a two-sided Student's *t*-test for single comparisons and by Bonferroni's test for multiple comparisons. Probability values of <0.05 were considered significant. Results are expressed as means \pm SEM.

RESULTS

Of the six PBC patients examined, two were Scheuer stage III, three were stage II, and one was stage I. The percentage of LDL present as Lp-X increased with the severity of the disease and was as high as 90% of total LDL in the stage III patients (Table 1). Because the bioactivity of PBC-LDL depends on its content of Lp-X (see below), we

TABLE 1. Characteristics and antioxidation index in six patients with primary biliary cirrhosis

Patient No.	Age	Sex	Scheuer Stage	Total Cholesterol	LDL Cholesterol	Lipoprotein-X Content of LDL	Plasma Bilirubin	Antioxidation Index
	years			mmol/l	mmol/l	%	$\mu\text{mol/l}$	
1	47	Female	III	34.1	31.7	90	222.3	5+
2	41	Female	III	31.7	28.4	88	201.8	5+
3	34	Female	II	29.3	27.1	65	78.7	4+
4	54	Female	II	10.1	7.5	ND	17.1	1+
5	41	Male	I	13.2	12.0	Trace	59.9	2+
6	43	Male	II	10.8	7.6	ND	20.5	1+

ND, not detectable. The antioxidation index was arbitrarily determined by the ability of a patient's LDL to inhibit the mobility of control LDL on agarose gel electrophoresis after 4 h of copper exposure ($5+ = 100\%$ inhibition).

describe the functional properties of PBC-LDL obtained from the patient with the highest Lp-X content. The patient was a 47 year old woman hospitalized in January 1999 for evaluation of intractable pruritus and generalized xanthomas. Serum values included antimitochondrial antibody titer of 1:160, alkaline phosphatase of 2,244 units/l, γ -glutamyl transpeptidase of 1,040 units/l, total bilirubin of 222.3 μ mol/l, total cholesterol of 34.1 mmol/l, and LDL-C of 31.7 mmol/l. Hepatobiliary scintigraphy and liver biopsy results were compatible with Scheuer stage III PBC. Despite marked hypercholesterolemia and widely distributed xanthomas, coronary angiographic and carotid ultrasonographic findings were normal. The patient denied symptoms of chest pain or intermittent claudication. A treadmill test was normal. Analysis of the patient's LDL by Superose 6HR 10/30 column chromatography showed that 90% of the patient's total LDL was accounted for by Lp-X, seen as an additional peak eluting in fractions 17–20 compared with a single peak in fractions 23–29 for control LDL (Fig. 1). None of the other five patients had a history of chest angina or peripheral symptoms that were ischemia related.

Exposure of the patient's total LDL fraction to copper for up to 18 h failed to induce a change in mobility on agarose gel electrophoresis (Fig. 2A), and TBARS remained <1 nmol/mg LDL protein (data not shown). In contrast, LDL isolated from a healthy subject was readily oxidized, exhibiting movement toward the anode by 2 h (Fig. 2A); TBARS concentration increased to 16 nmol/mg at 18 h. Equal-concentration (200 μ g/ml) mixing with the pa-

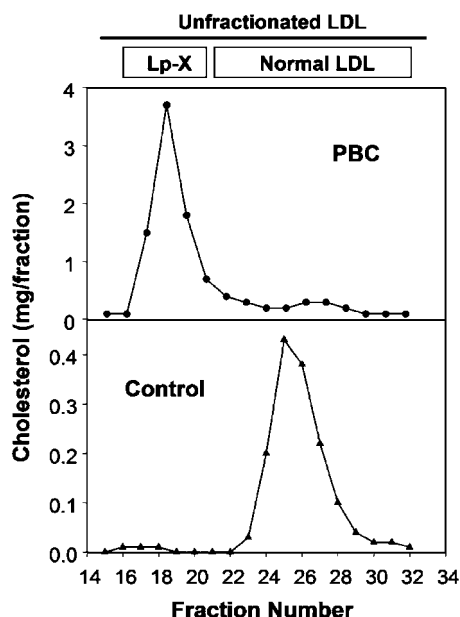


Fig. 1. Gel filtration chromatography of unfractionated LDL from plasma of a patient and a healthy subject (control). Lipoproteins of $1.019 < d < 1.063$ isolated from 1 ml of plasma were applied to a Superose 6HR 10/30 column at a flow rate of 0.25 ml/min and collected 0.5 ml/tube. The fractions of lipoprotein-X (Lp-X) and normal LDL are indicated. Experiments using primary biliary cirrhosis (PBC)-LDL samples obtained from the other five patients yielded similar results to varying degrees, depending on the Lp-X content.

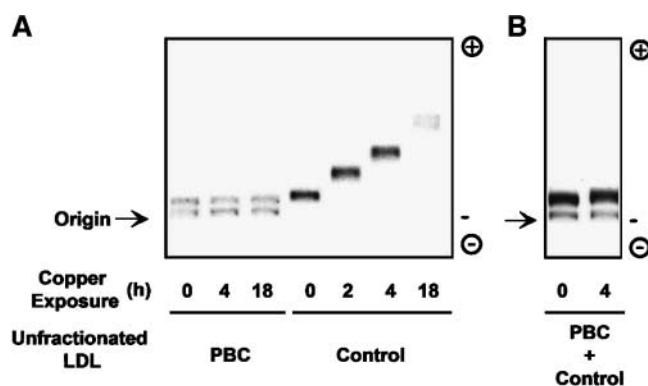


Fig. 2. LDL oxidation assessed by agarose gel electrophoresis of lipoproteins from a patient with stage III PBC and a healthy subject (control). A: Patient and control unfractionated LDLs (200 μ g/ml) were incubated with 10 μ M CuSO_4 for 0–18 h as indicated. B: Equal-concentration mixing of patient and control unfractionated LDLs. The assay procedures are described in the text. Experiments using PBC-LDL samples obtained from the other five patients yielded similar results to varying degrees, depending on the Lp-X content.

tient's unfractionated LDL suppressed control LDL oxidizability and completely prevented a change in control LDL mobility (Fig. 2B). The extent of inhibition in electrophoretic mobility by PBC-LDL correlated well with the PBC-LDL molar ratio to control LDL. As shown in Fig. 3, an admixture of PBC-LDL and control LDL at 1:9, 1:4, or 1:1 exhibited a dose-response effect on the mobility change after exposure to copper for 8 h (Fig. 3). To determine the antioxidant properties of the subfractions of PBC-LDL, electrophoretic mobility was assessed in the Lp-X (fractions 17–20) and the normal LDL (fractions 23–29) from the same patient. At 2 h of copper exposure, the patient's Lp-X was resistant to oxidation, whereas her normal LDL exhibited electrophoretic mobility changes proportional to the duration of exposure (Fig. 4A). Her normal LDL

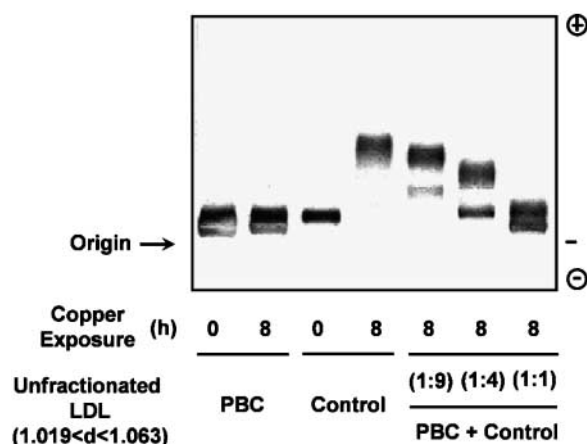


Fig. 3. Dose-dependent effect of patient LDL on LDL oxidation. Patient and control unfractionated LDLs were mixed at 1:9, 1:4, or 1:1 ratio as indicated and incubated with 10 μ M CuSO_4 for 8 h. LDL oxidation was assessed by agarose gel electrophoresis. Experiments using PBC-LDL samples obtained from other patients yielded similar results to varying degrees, depending on the Lp-X content.

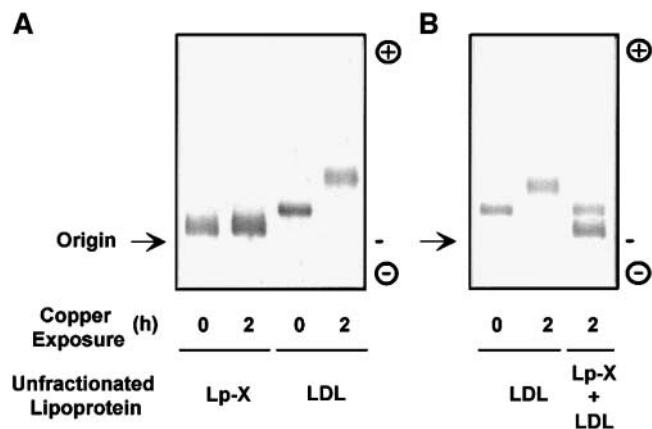


Fig. 4. Oxidation analysis of isolated Lp-X and LDL of a PBC patient. A: Patient Lp-X (fractions 17–20) and normal LDL (fractions 23–29) were incubated with 10 μ M CuSO₄ for 0–2 h as indicated followed by agarose gel electrophoresis. B: Equal-concentration mixing of patient Lp-X and normal LDL fractions incubated with 10 μ M CuSO₄ for 0–2 h as indicated.

fraction became completely oxidation resistant after admixture with an equal amount of her Lp-X (Fig. 4B). Using the ability of PBC-LDL to attenuate the electrophoretic mobility as an index, we found that such an ability depended on the content of Lp-X (Table 1). In both Scheuer stage II patients, the PBC-LDL had a marked antioxidant effect, which was negligible in PBC-LDL with minimal Lp-X. Of note, although plasma concentrations of bilirubin, a potent antioxidant, increased as Lp-X levels increased (Table 1), bilirubin was not detected in Lp-X.

The oxidizability of patient LDL was also assessed by competitive ELISA using the anti-oxLDL monoclonal antibody mAb-4E6. Copper-treated patient LDL at more than 1,000-fold higher molar concentrations failed to compete with oxLDL for binding (Fig. 5). The binding capability of patient LDL was comparable to that of control LDL, confirming an oxidation-resistant feature of Lp-X.

Endothelial survival was measured by DNA synthesis and FGF2 expression in cultured BAECs. Treatment of BAECs with oxLDL produced concomitant reductions in intracellular FGF2 peptide levels and cell proliferation. Unlike control LDL (50 μ g/ml), PBC-LDL (50 μ g/ml) incubated with copper for up to 24 h failed to reduce the FGF2 protein concentration. Preincubation of oxLDL (50 μ g/ml) with PBC-LDL attenuated FGF2 reduction in a dose-dependent manner, whereas preincubation with control LDL did not alter the inhibitory effect of oxLDL (Fig. 6A). The FGF2 mRNA level was also reduced by oxLDL but not by PBC-LDL. Preincubation of oxLDL with PBC-LDL also attenuated oxLDL-induced FGF2 mRNA down-regulation (Fig. 6B). In accordance with the changes in FGF2 expression, oxLDL decreased DNA synthesis by half, whereas PBC-LDL had no effect. Preincubation of oxLDL with PBC-LDL, but not control LDL, prevented the inhibitory effect of oxLDL (data not shown).

After the patient received an orthotopic liver transplant in March 1999, her total cholesterol decreased within 2

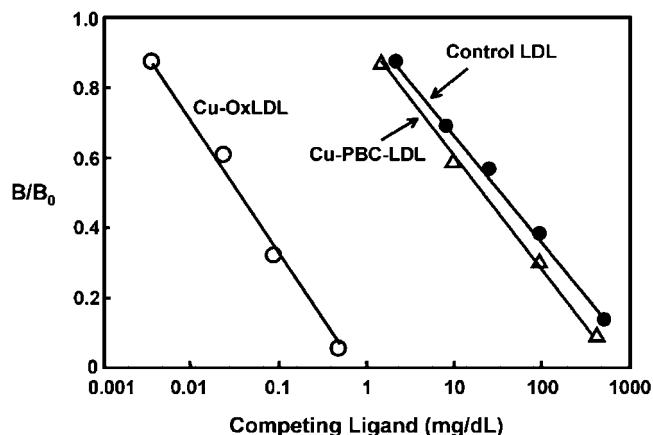


Fig. 5. Evaluation of the oxidizability of LDL by ELISA. A competitive ELISA was used to measure the oxidizability of LDL using the copper-oxidized LDL (oxLDL)-specific monoclonal antibody mAb-4E6 according to the protocols described in the text. Competing ligands included oxLDL, copper-treated PBC-LDL (Cu-PBC-LDL), and control LDL as indicated. B and B₀ represent absorbance in the presence and absence of competitor, respectively. Experiments using PBC-LDL samples obtained from the other five patients yielded similar results to varying degrees, depending on the Lp-X content.

weeks to 2.6 mmol/l, Lp-X disappeared, and the LDL no longer exerted antioxidant action (data not shown). In contrast to her pretransplantation LDL, which failed to induce a change in mobility after copper oxidation (Fig. 2A), her posttransplantation LDL exhibited electrophoretic mobility changes proportional to the duration of copper exposure. The oxidizability was not different from that of control LDL. Admixing control LDL with patient LDL no longer attenuated the mobility of control LDL (data not shown).

DISCUSSION

Hypercholesterolemic human plasma contains atherogenic LDL subfractions that can inhibit proliferation and increase apoptosis in ECs and induce monocyte-EC adhesion (9, 19). However, despite the highly increased cholesterol levels and marked xanthomas commonly found in patients with PBC (2, 3), the incidence of atherosclerotic cardiovascular risk does not increase in these patients (4). Our findings indicate that LDL isolated from the plasma of PBC patients not only protected normolipidemic LDL from becoming oxidized but also prevented oxLDL from disrupting the FGF2-dependent survival mechanism in vascular ECs. The antioxidant effect of PBC-LDL depended on its Lp-X content. These findings may further substantiate the LDL oxidation hypothesis for the pathogenesis of atherosclerosis.

The atherogenic properties of LDL have been postulated to result from oxidative modification of the lipoprotein (6, 20–22). Clinically, the incidence of acute coronary events is related to the concentration of circulating oxLDL detectable by immunoassay (23, 24). Electronegative LDL is a naturally occurring, mildly oxidized LDL species

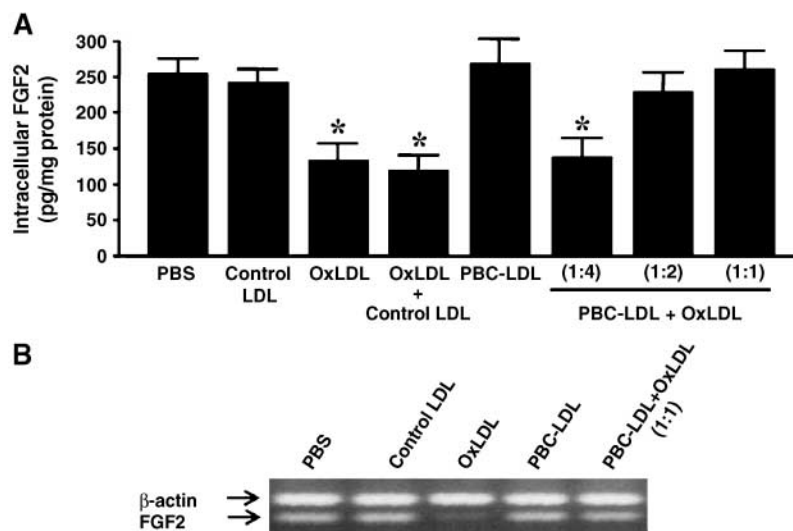


Fig. 6. Effects of LDL on intracellular fibroblast growth factor 2 (FGF2) expression. A: FGF2 peptide levels measured by ELISA. Bovine aortic endothelial cells (BAECs) were incubated with different LDL preparations (50 μ g/ml each) as indicated for 24 h before ELISA, according to the protocols described in the text. Values shown are means \pm SEM ($n = 3$). Each well contained 1×10^6 cells at inoculation. * $P < 0.05$ vs. PBS control. B: FGF2 mRNA measured by RT-PCR. BAECs were incubated with different LDL preparations (50 μ g/ml each) as indicated for 24 h, and isolated total RNA was subjected to RT-PCR analysis as described in the text. Data are representative of three separate experiments with similar results.

that can induce a variety of atherogenic effects in vascular ECs (9, 19, 25). Because electronegative LDL is more abundant in hypercholesterolemic than normolipidemic human plasma (9), hypercholesterolemic LDL should have a greater susceptibility to oxidation than normolipidemic LDL. Failure of hypercholesterolemia to increase atherosclerosis incidence in PBC raised the question of whether PBC-LDL is resistant to oxidative modification.

Lipid peroxidation-producing products, including 4-hydroxynonenal, are considered important in the pathologic process leading to the bile duct damage of PBC (26). In early PBC, lipid peroxidation markers, including plasma and urinary 8-isoprostane and plasma malondialdehyde, are increased, whereas plasma antioxidants, such as total glutathione, are decreased (27). Although these changes lead to eventual bile duct pathology, their effects on the vascular wall have not been documented. In this report, severe biliary cirrhosis and extensive occurrence of xanthomas were not accompanied by coronary or carotid narrowing.

Previous reports have attributed the paradoxically low atherosclerosis incidence in PBC to high plasma concentrations of HDL and Lp-A-I (28) and the observation that most patients were premenopausal women (2). Although the contribution of these protective factors cannot be discounted, premature coronary disease in patients with LDL-C increased to the degree seen in PBC patients is well documented in non-PBC patients regardless of their overall lipid profile, age, or gender. Our data show that PBC-LDL was resistant to copper oxidation, probably because of the high rigidity of the particle, thereby preventing penetration of copper ions into the hydrophobic region of the bilayer, where oxidizable unsaturation sites are localized. Even more important, the Lp-X fraction isolated from PBC-LDL ren-

dered the non-Lp-X fraction from the same origin resistant to copper oxidation. This may have been attributable to the higher affinity of Lp-X than LDL for copper ions. The capability of PBC-LDL to suppress oxidation-induced change in the electrophoretic mobility of control LDL appeared to reflect the Lp-X/LDL concentration ratio. These findings provide a new explanation for vascular protection in PBC other than the HDL, age, and gender parameters. Bilirubin, a potent physiological antioxidant in plasma, has been shown to inhibit LDL oxidation in vitro (29). The concomitant increase in plasma bilirubin with Lp-X may play an additional role in shielding LDL from oxidation in patients with PBC. However, the antioxidant effect of Lp-X observed in this study was independent of bilirubin, because there was a lack of detectable bilirubin in these Lp-X fractions.

In this study, copper ion was used as an initiating agent for LDL oxidation. Copper-mediated LDL oxidation is the most common means of initiating LDL oxidation in vitro (30), and the copper-catalyzed processes are of potential physiological relevance. Studies have shown that oxidized products of LDL induced by copper are similar to those occurring in the presence of cells (13), and the biological properties of oxLDL are very similar to those of atherogenic LDL isolated from hypercholesterolemic human plasma (9, 19). Furthermore, atheromatous lesions have been shown to contain copper ion in forms that can catalyze free radical formation (31). The role of copper ion as a catalyst in LDL oxidation may be limited by existing metal-chelating agents such as thiols that can inhibit copper-dependent LDL oxidation in vivo (32, 33). In our study, however, it is unlikely that the antioxidant activity of Lp-X was caused by copper ion chelation, because Lp-X lost its antioxidant ac-

tivity gradually when unfractionated patient LDL was stored at 4°C and during gel-filtration purification of Lp-X (data not shown).

FGF2 is a pleiotropic protein that exerts angiogenic and antiapoptotic effects by activating the phosphatidylinositol 3-kinase-Akt system and the subsequent signaling pathways (7, 8). Both oxLDL and electronegative LDL down-regulate FGF2 expression in ECs, contributing to reduced proliferation and increased apoptosis (9–11). In this study, PBC-LDL did not inhibit FGF2 expression or reduce DNA synthesis in BAECs. Moreover, it completely prevented the inhibitory effects of oxLDL on these parameters. This attenuation cannot be explained by dilution of oxLDL after 1:1 admixture, because control LDL failed to attenuate oxLDL's effects after a similar admixture.

These findings indicate that PBC-LDL protected ECs in two ways. First, it prevented the oxidation of normal LDL, as confirmed by Lp-X's protection of the Lp-X-free part of LDL from the same individual against oxidation. Second, PBC-LDL effectively attenuated the adverse effects of LDL that had already been oxidized on ECs. The second protective mechanism is not clear at this time. Because hydrolyzing platelet-activating factor or platelet-activating factor-like lipids accumulated in oxLDL abolishes the bioactivities of the lipoprotein (11), one possibility is that PBC-LDL or Lp-X is rich in phospholipases that can degrade these lipids in oxLDL. Further experiments are required to test this possibility.

The striking disappearance of the protection of the PBC patient's LDL from oxidation after liver transplantation further confirmed the antioxidant role of PBC-LDL attributable to its Lp-X components. These results are not consistent with the view that the low incidence of atherosclerosis in PBC is primarily a consequence of concomitant increases in HDL and Lp-A-I.

In conclusion, our findings indicate that PBC-LDL protects EC integrity by preventing LDL oxidation and the adverse effects of LDL with any degree of oxidative modification that may have occurred in vivo. Although the mechanism is not completely elucidated at this time, the antioxidant effect of PBC-LDL can be attributed to its Lp-X component. The findings also suggest that changing the composition of LDL to render it resistant to oxidants may be as important as decreasing the plasma concentration of LDL in the prevention or treatment of atherosclerosis. **FIG**

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REFERENCES

1. National Cholesterol Education Program. 2002. Third report of the National Cholesterol Education Program (NCEP) Expert Panel

- on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation*. **106**: 3143–3421.
2. Kaplan, M. M. 1996. Primary biliary cirrhosis. *N. Engl. J. Med.* **335**: 1570–1580.
3. Jahn, C. E., E. J. Schaefer, L. A. Taam, J. H. Hoofnagle, F. T. Lindgren, J. J. Albers, E. A. Jones, and H. B. Brewer. 1985. Lipoprotein abnormalities in primary biliary cirrhosis: association with hepatic lipase inhibition as well as altered cholesterol esterification. *Gastroenterology*. **89**: 1266–1278.
4. Crippin, J. S., K. D. Lindor, R. Jorgensen, B. A. Kottke, J. M. Harrison, P. A. Murtaugh, and E. R. Dickson. 1992. Hypercholesterolemia and atherosclerosis in primary biliary cirrhosis: what is the risk? *Hepatology*. **15**: 858–862.
5. Patsch, J. R., K. C. Aune, A. M. Gotto, Jr., and J. D. Morrisett. 1997. Isolation, chemical characterization, and biophysical properties of three different abnormal lipoproteins: LP-X1, LP-X2, and LP-X3. *J. Biol. Chem.* **252**: 2113–2120.
6. Steinberg, D., and J. L. Witztum. 2002. Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? *Circulation*. **105**: 2107–2111.
7. Raffioni, S., and R. A. Bradshaw. 1992. Activation of phosphatidylinositol 3-kinase by epidermal growth factor, basic fibroblast growth factor, and nerve growth factor in PC12 pheochromocytoma cells. *Proc. Natl. Acad. Sci. USA*. **89**: 9121–9125.
8. Chen, C. H., S. M. Poucher, J. Liu, and P. D. Henry. 2004. Fibroblast growth factor 2: from laboratory evidence to clinical application. *Curr. Vasc. Pharmacol.* **2**: 33–43.
9. Chen, C. H., T. Jiang, J. H. Yang, W. Jiang, J. Lu, G. K. Marathe, H. J. Pownall, C. M. Ballantyne, T. M. McIntyre, P. D. Henry, and C. Y. Yang. 2003. Low-density lipoprotein in hypercholesterolemic human plasma induces vascular endothelial cell apoptosis by inhibiting fibroblast growth factor 2 transcription. *Circulation*. **107**: 2102–2108.
10. Chen, C. H., W. Jiang, D. P. Via, S. Luo, T. R. Li, Y. T. Lee, and P. D. Henry. 2000. Oxidized low-density lipoproteins inhibit endothelial cell proliferation by suppressing basic fibroblast growth factor expression. *Circulation*. **101**: 171–177.
11. Chang, P. Y., S. Luo, T. Jiang, Y. T. Lee, S. C. Lu, P. D. Henry, and C. H. Chen. 2001. Oxidized low-density lipoprotein down-regulates endothelial bFGF through a pertussis toxin-sensitive G-protein pathway: mediator role of platelet-activating factor-like phospholipids. *Circulation*. **104**: 588–593.
12. Franceschini, G., G. Busnach, G. Chiesa, and C. R. Sirtori. 1991. Management of lipoprotein-X accumulation in severe cholestasis by semi-selective LDL-apheresis. *Am. J. Med.* **90**: 633–638.
13. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witztum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA*. **81**: 3883–3887.
14. Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83**: 346–356.
15. Lu, S. C., W. H. Wu, C. A. Lee, H. F. Chou, H. R. Lee, and P. C. Huang. 2000. LDL of Taiwanese vegetarians are less oxidizable than those of omnivores. *J. Nutr.* **130**: 1591–1596.
16. Puhl, H., G. Wang, and H. Esterbauer. 1994. Methods to determine oxidation of low-density lipoproteins. *Methods Enzymol.* **233**: 425–441.
17. Holvoet, P., J. Vanhaecke, S. Janssens, F. Van de Werf, and D. Collen. 1998. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation*. **98**: 1487–1494.
18. Chen, C. H., J. Cartwright, Jr., Z. Li, S. Luo, H. H. Nguyen, A. M. Gotto, Jr., and P. D. Henry. 1997. Inhibitory effects of hypercholesterolemia and oxidized LDL on angiogenesis-like endothelial growth in rabbit aortic explants: essential role of basic fibroblast growth factor. *Arterioscler. Thromb. Vasc. Biol.* **17**: 1303–1312.
19. Yang, C. Y., J. L. Raya, H. H. Chen, C. H. Chen, Y. Abe, H. J. Pownall, A. A. Taylor, and C. V. Smith. 2003. Isolation, characterization, and functional assessment of oxidatively modified subfractions of circulating low-density lipoproteins. *Arterioscler. Thromb. Vasc. Biol.* **23**: 1083–1090.
20. Shaw, P. X., S. Horkko, S. Tsimikas, M. K. Chang, W. Palinski, G. J. Silverman, P. P. Chen, and J. L. Witztum. 2001. Human-derived

- anti-oxidized LDL autoantibody blocks uptake of oxidized LDL by macrophages and localizes to atherosclerotic lesions in vivo. *Arterioscler. Thromb. Vasc. Biol.* **21**: 1333–1339.
21. Walton, K. A., X. Hsieh, N. Gharavi, S. Wang, G. Wang, M. Yeh, A. L. Cole, and J. A. Berliner. 2003. Receptors involved in the oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine-mediated synthesis of interleukin-8. A role for Toll-like receptor 4 and a glycosylphosphatidylinositol-anchored protein. *J. Biol. Chem.* **278**: 29661–29666.
22. Marathe, G. K., G. A. Zimmerman, and T. M. McIntyre. 2003. Platelet-activating factor acetylhydrolase, and not paraoxonase-1, is the oxidized phospholipid hydrolase of high density lipoprotein particles. *J. Biol. Chem.* **278**: 3937–3947.
23. Holvoet, P., D. Collen, and F. Van de Werf. 1999. Malondialdehyde-modified LDL as a marker of acute coronary syndromes. *J. Am. Med. Assoc.* **281**: 1718–1721.
24. Holvoet, P., T. B. Harris, R. P. Tracy, P. Verhamme, A. B. Newman, S. M. Rubin, E. M. Simonsick, L. H. Colbert, and S. B. Kritchevsky. 2003. Association of high coronary heart disease risk status with circulating oxidized LDL in the well-functioning elderly: findings from the Health, Aging, and Body Composition Study. *Arterioscler. Thromb. Vasc. Biol.* **23**: 1444–1448.
25. Ziouzenkova, O., L. Asatryan, D. Sahady, G. Orasanu, S. Perrey, B. Cutak, T. Hassell, T. E. Akiyama, J. P. Berger, A. Sevanian, and J. Plutzky. 2003. Dual roles for lipolysis and oxidation in peroxisome proliferation-activator receptor responses to electronegative low density lipoprotein. *J. Biol. Chem.* **278**: 39874–39881.
26. Tsuneyama, K., K. Harada, N. Kono, M. Sasaki, T. Saito, M. E. Gershwin, M. Ikemoto, H. Arai, and Y. Nakanuma. 2002. Damaged interlobular bile ducts in primary biliary cirrhosis show reduced expression of glutathione-S-transferase-pi and aberrant expression of 4-hydroxynonenal. *J. Hepatol.* **37**: 176–183.
27. Aboutwerat, A., P. W. Pemberton, A. Smith, P. C. Burrows, R. F. McMahon, S. K. Jain, and T. W. Warnes. 2003. Oxidant stress is a significant feature of primary biliary cirrhosis. *Biochim. Biophys. Acta.* **1637**: 142–150.
28. O'Kane, M. J., P. L. M. Lynch, M. E. Callender, and E. R. Trimble. 1997. Abnormalities of serum apo A1 containing lipoprotein particles in patients with primary biliary cirrhosis. *Atherosclerosis.* **131**: 203–210.
29. Neuzil, J., and R. Stocker. 1994. Free and albumin-bound bilirubin are efficient co-antioxidants for alpha-tocopherol, inhibiting plasma and low density lipoprotein lipid peroxidation. *J. Biol. Chem.* **269**: 16712–16719.
30. Burkitt, M. J. 2001. A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: roles of lipid hydroperoxides, alpha-tocopherol, thiols, and ceruloplasmin. *Arch. Biochem. Biophys.* **394**: 117–135.
31. Smith, C., M. J. Mitchinson, O. I. Aruoma, and B. Halliwell. 1992. Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. *Biochem. J.* **286**: 901–905.
32. Lynch, S. M., and B. Frei. 1995. Reduction of copper, but not iron, by human low density lipoprotein (LDL). Implications for metal ion-dependent oxidative modification of LDL. *J. Biol. Chem.* **270**: 5158–5163.
33. Lynch, S. M., and B. Frei. 1997. Physiological thiol compounds exert pro- and anti-oxidant effects, respectively, on iron- and copper-dependent oxidation of human low-density lipoprotein. *Biochim. Biophys. Acta.* **1345**: 215–221.