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Oxidation Resistance of LDL in Hypertriglyceridaemic Patients Treated With Ciprofibrate

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Summary

The oxidative modification of low density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis. LDL of subjects with atherogenic lipoprotein phenotype (ALP) is known to be more susceptible to oxidation. We studied the effect of the hypolipidaemic drug ciprofibrate on the susceptibility of LDL to *in vitro* oxidation. Nine patients with primary hypertriglyceridaemia and hypoalphalipoproteinaemia (mean plasma triglycerides 3.76 $mmol.l^{-1}$ and HDL-cholesterol 0.74 $mmol.l^{-1}$) were treated with ciprofibrate for 12 weeks. The susceptibility of LDL to *in vitro* Cu^{2+}-mediated oxidation was assessed by measuring conjugated diene formation at 234 nm. Ciprofibrate therapy significantly prolonged the lag time (93±7 min vs. 102±11 min, P=0.02). The maximal rate of diene production was 11 % lower, but the decrease was not significant. A significant positive correlation was observed between maximal rate and maximal amount of dienes formed. Thiobarbituric acid reacting substances (TBARS) and lipid hydroperoxides (LPO) in oxidatively-modified LDL, isolated from the plasma of patients before and after drug treatment, were unchanged. The results suggest that ciprofibrate therapy has a favourable effect by increasing the *in vitro* resistance of LDL against oxidation.

Key words
Atherosclerosis – Ciprofibrate – LDL – Oxidative modification

Introduction

The oxidative modification of low density lipoprotein (LDL) is a key event in early atherogenesis (Steinberg *et al.* 1989). Oxidatively-modified LDL is taken up by scavenger receptors of macrophages in an uncontrolled fashion, leading to the production of lipid-laden foam cells (Steinbrecker *et al.* 1984, Parthasarathy *et al.* 1987). The formation of atherogenic oxidized LDL (ox-LDL) can be inhibited by dietary antioxidants such as vitamins C and E, carotenoids and phenolic compounds (Sato *et al.* 1990, Lavy *et al.* 1993). Some lipid lowering drugs (namely statins) have also been shown to reduce the susceptibility of LDL to oxidation in hyperlipidaemic patients (Hoffman *et al.* 1992, Kleinveld *et al.* 1993, Hussein *et al.* 1997).

Ciprofibrate is a lipid-lowering drug, belonging to the class of fibric acid derivates. Its beneficial effects on plasma very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein (HDL) and on the metabolism of LDL have been documented (Oro *et al.* 1992, Gaw *et al.* 1994). Ciprofibrate also normalized both the quantitative and qualitative features about the atherogenic LDL particle profile in combined hyperlipidaemia (Bruckert *et al.* 1993, Chapman and Bruckert 1996). To the best of our knowledge, there have been no reports of the effect of ciprofibrate on LDL susceptibility to *in vitro* oxidation.

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Therefore, we studied the effect of ciprofibrate treatment for 12 weeks on the *in vitro* resistance to Cu^{2+}-induced oxidation of LDL isolated from the plasma of patients with the atherogenic lipoprotein phenotype (hypertriglyceridaemia and hypoalpha-lipoproteinaemia). The extent of oxidative modification was measured by the kinetics of conjugated diene formation, thiobarbituric acid reacting substances (TBARS) and lipid hydroperoxides (LPO).

Materials and Methods

Thirteen patients with atherogenic lipoprotein phenotype (hypertriglyceridaemia and hypoalpha-lipoproteinaemia) entered the prospective open trial after a 6-week wash-out period on a low fat-low cholesterol diet (less than 200 mg of cholesterol per day). The patients had neither any disorder causing secondary hyperlipoproteinaemia, nor a recent history of acute myocardial infarction, stroke, surgery, unstable angina pectoris or gastroduodenal ulcer. Ciprofibrate (Lipanor 100) was taken in one capsule (100 mg) once a day for 12 weeks. Three patients dropped out from the study after six weeks of ciprofibrate treatment (one patient stopped the treatment during influenza infection and two patients refused further cooperation). The results of the tenth patient were not evaluated.

Blood samples, after an overnight fast, were collected at the start and after 12 weeks of drug treatment into EDTA-containing tubes and centrifuged for 15 min at 3000 rpm. Total cholesterol and triglycerides were determined enzymatically, apolipoprotein B and AI were measured using an immunoturbidimetric method (Boehringer-Mannheim, autoanalyzer HITACHI 911). HDL-cholesterol was measured by dextran sulphate precipitation followed by enzymatic determination of cholesterol. Plasma samples, to which saccharose (final concentration 6 $g.l^{-1}$) and butylated hydroxytoluene (BHT; final concentration 4.4 $mg.l^{-1}$) had been added, were frozen at −80 °C (Kleinveld *et al.* 1992) and analyzed for LDL-resistance simultaneously at the end of the drug treatment.

LDL (d = 1.019 − 1.063 $kg.l^{-1}$) was isolated by density gradient ultracentrifugation (Terpstra *et al.* 1981) in a Beckman L7-65 ultracentrifuge at 15 °C. The LDL fraction, located in the centre of the tube, was collected by aspiration and extensively dialyzed in the dark for 15 h at 4 °C against 0.01 $mol.l^{-1}$ phosphate buffer (pH = 7.4) containing 0.16 $mol.l^{-1}$ NaCl (PBS). The buffer was prepared oxygen-free by intensive purging with nitrogen. The protein content was determined in LDL according to Lowry *et al.* (1951).

The kinetics of oxidation of the LDL fraction were determined according to the method of Esterbauer *et al.* (1989) by measuring conjugated diene formation. Briefly, dialyzed LDL was diluted directly before the start of oxidation with an EDTA-free phosphate buffer to a final concentration of 50 $\mu g.ml^{-1}$. Oxidation was initiated by addition of freshly prepared $CuCl_2$ (final concentration in the cuvette 1.66 μM). LDL oxidation kinetics were monitored by the change in 234 nm absorbance at 30 °C every 5 min for 4 h on a Beckman spectrophotometer DU-650 equipped with a six-position automatic sample changer. To describe the oxidizability of LDL fractions we used five parameters from the oxidation curve (Kleinveld *et al.* 1992): the lag time defined as the time between the intercept of the linear least-square slope of the curve with the horizontal axis (lag time), the maximal rate of oxidation (V_{max}) at time t, the maximal amount of dienes formed (CD_{max}) and the time needed to reach maximal diene production (t_{max}) (Fig. 1). V_{max} and CD_{max}, expressed as nanomoles of dienes produced per minute per mg of LDL-protein and nanomoles per mg of LDL-protein, respectively, were calculated using the molar absorbance coefficient for conjugated dienes (ϵ_{234} = 29 500).

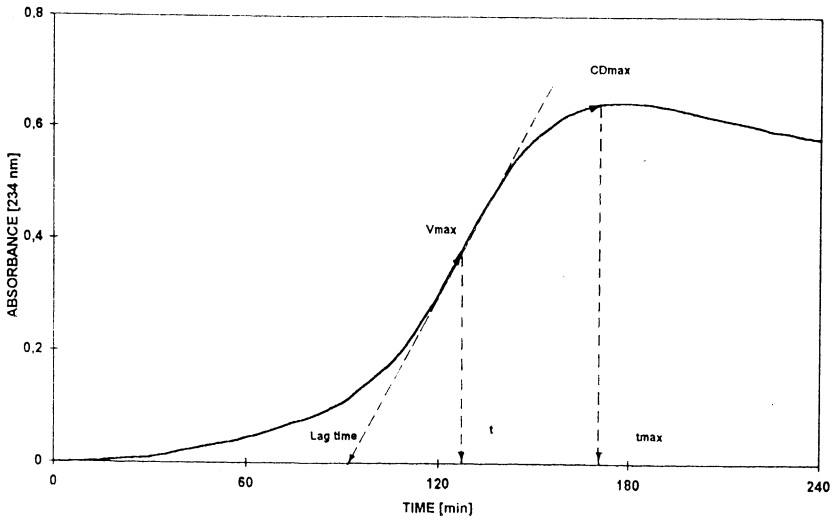

Fig. 1. *Kinetics of LDL oxidation determined by monitoring the change in absorbance at 234 nm. From this curve five parameters were determined: lag time, maximal rate of oxidation (V_{max}) at the time t, maximal amount of dienes formed (CD_{max}) and the time needed to reach maximal diene production (t_{max}).*

LDL oxidation for TBARS (thiobarbituric acid reacting substances) determination was performed under the following conditions: 0.3 mg of LDL-protein was diluted in 3 ml of PBS and incubated in the presence of $CuSO_4$ (final concentration 10 μM) at 37 °C for 4 h. At the times 0 and 4 h the oxidation was stopped by refrigeration and addition of EDTA (final concentration 0.2 mM) and BHT (final concentration 40 μM). TBARS were measured using the method of Buege and Aust (1978). The concentration of malondialdehyde (MDA) was calculated by using the extinction coefficient for MDA (1.56×10^5 $l.mol^{-1}.cm^{-1}$).

For the determination of lipid hydroperoxides (LPO), 0.3 mg of LDL-protein in 0.6 ml of PBS in the presence of $CuCl_2$ (final concentration 5 μM) were incubated at 37 °C for 4 h. The reaction was stopped by refrigeration and addition of EDTA and BHT (final concentrations as for TBARS). LPO were estimated iodometrically according to El-Saadani *et al.* (1989) and the concentration was calculated using the molar extinction coefficient 2.46×10^{-4} $mol.l^{-1}.cm^{-1}$. The length of incubation for both TBARS and LPO corresponds to the maximum of their concentrations, as determined in our previous experiments. TBARS and LPO determinations were performed in duplicates.

The results, presented as means ± S.D. were statistically analyzed using Student's paired t-test or the Wilcoxon test. Correlation analysis was performed by Spearman correlation analysis (Statgraphics). The minimal acceptable level of significance was $P<0.05$.

Table 1. Effect of ciprofibrate therapy on plasma lipids and lipoproteins

	Ciprofibrate therapy	
	Before	After
Total cholesterol ($mmol.l^{-1}$)	5.75±1.01	5.01±0.91**
Triglycerides ($mmol.^{-1}$)	3.76±1.61	1.98±0.81***
HDL-cholesterol ($mmol.l^{-1}$)	0.74±0.14	0.90±0.19***
Apo-B ($g.l^{-1}$)	0.91±0.15	0.77±0.13*
Apo-AI ($g.l^{-1}$)	1.04±0.14	1.17±0.22

*Values are means ± S.D., * $P<0.05$, ** $P<0.01$, *** $P<0.001$.*

Results

The effect of therapy with the hypolipidaemic drug ciprofibrate on *in vitro* oxidation of LDL was studied in nine patients with primary hypertriglyceridaemia and hypoalphalipoproteinaemia (ALP) before and 12 weeks after treatment. Ciprofibrate therapy resulted in significant decreases in plasma total cholesterol, triglycerides and apolipoprotein B (13 %, 47 % and 15 %, respectively) and an increase in HDL-cholesterol (22 %) (Table 1).

Table 2. Effect of the therapy with ciprofibrate for 12 weeks on LDL oxidizability parameters

Parameter	Ciprofibrate therapy	
	Before	After
Lag time (min)	93±7	102±11*
Maximal rate,		
V_{max} (nmol/min/mg LDL protein)	6.29±1.15	5.60±1.07
at time t (min)	131±9	138±15
Maximal dienes,		
CD_{max} (nmol/mg LDL protein)	370±52	356±69
at time t_{max} (min)	170±16	187±15*

*Values are means ±S.D. * $P<0.05$.*

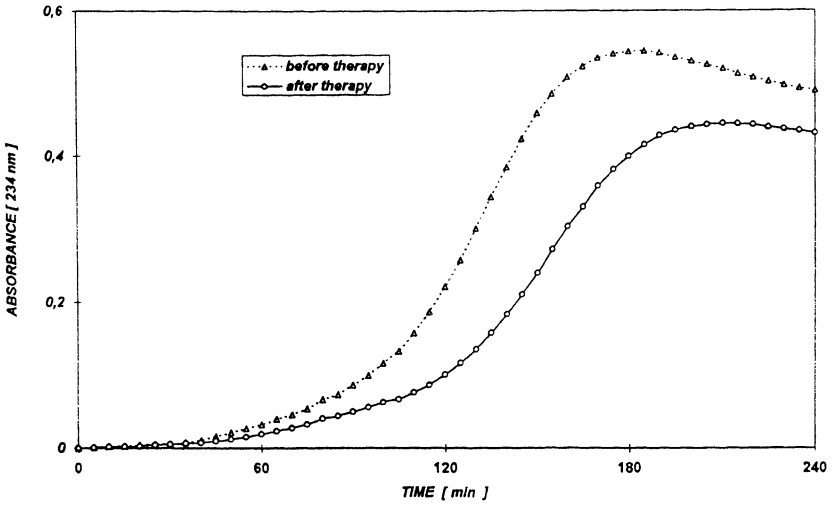

Fig. 2. *Kinetics of conjugated diene formation during Cu^{2+}-mediated oxidation of LDL isolated from hypertriglyceridaemic patient before and after ciprofibrate therapy. (open triangles) vs. (open circles) 95 and 112 (lag time); 5.84 and 4.30 (maximal rate) and 368 and 300 (diene max.).*

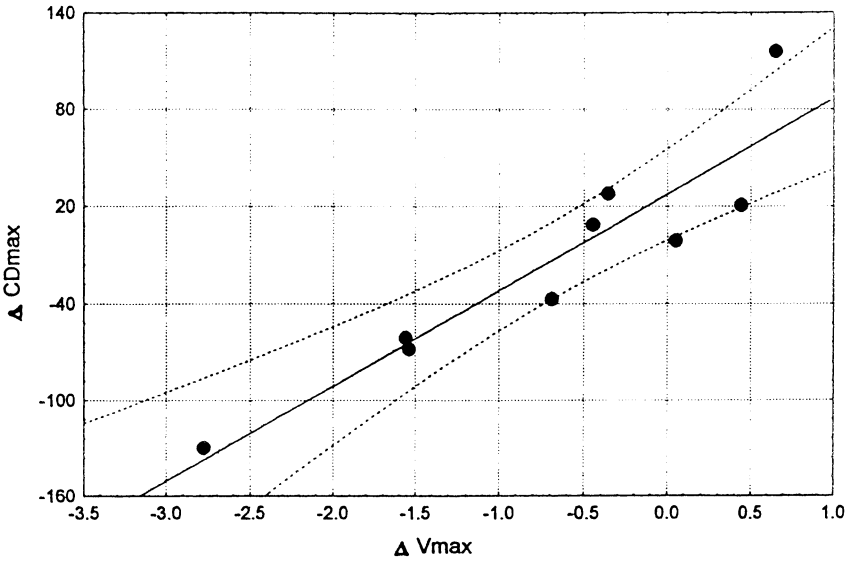

Fig. 3. *Correlation between maximal rate of diene production (V_{max}) and maximal amount of dienes formed (CD_{max}). The difference in mean values before and after therapy from each patient ($r=0.925$, $P<0.05$).*

Table 3. Conjugated dienes (CD), thiobarbituric acid reacting substances (TBARS) and lipid hydroperoxides (LPO) concentrations in LDL after 4-hour oxidation

	Ciprofibrate therapy	
	Before	After
CD (nmol/mg LDL protein)	334±44	332±64
TBARS (nmol/mg LDL protein)	53.7±8.7	55.9±10.2
LPO (nmol/mg LDL protein)	436±85	425±81

Values are means ±SD. CD values were calculated from the kinetic curve for CD formation.

The resistance of LDL to oxidation, as measured by the duration of the lag time during copper-mediated oxidation, was greater in the patients after ciprofibrate therapy (93±7 min vs. 102±11 min, P=0.02) (Table 2, Fig. 2). The maximal rate of diene production during *in vitro* oxidation of LDL decreased by 11 % (decrease in 6 patients, no change in 3 patients) but this decrease was not significant. Despite that, a significant positive correlation between maximal rate and maximal amount of dienes formed was observed (Fig. 3). The maximal amount of dienes formed during LDL oxidation was not significantly affected by ciprofibrate therapy.

LDL oxidation at the maximum point was also determined by assaying TBARS and LPO after 4 h of *in vitro* oxidation. No significant differences were seen between LDL samples taken from patients before and after drug therapy (Table 3).

Discussion

The present study has demonstrated that ciprofibrate therapy, in addition to its beneficial effects on plasma lipoproteins, increased the resistance of LDL to oxidation as measured by the duration of the lag time. Different hypolipidaemic drugs have been shown to have different effects on the *in vitro* resistance of LDL to oxidation. The reduced susceptibility of LDL to oxidation in hypercholesterolaemic patients treated with fluvastatin for 24 weeks was demonstrated (Hussein *et al.* 1997). The authors supposed that the antiatherogenic properties of fluvastatin are not limited to its hypocholesterolaemic effect, but could also be related to its ability to reduce LDL oxidizability. The same authors also demonstrated that hypolipidaemic drugs such as pravastatin, cholestyramine and bezafibrate reduce the *ex vivo* susceptibility of LDL to oxidation in hypercholesterolaemic patients (Hoffman *et al.* 1992). Cholestyramine and bezafibrate were more potent than pravastatin in this respect. One of their explanations for the reduced propensity for lipoprotein oxidation after treatment with hypolipidaemic drugs, is that the LDL population contained less aged particles than could be found prior to drug treatment. Hypocholesterolaemic drugs induce the enrichment of plasma LDL with newly formed lipoprotein as a result of the liver response to plasma LDL reduction.

Fibrates significantly alter the composition of LDL (lipid and protein components) (Goldberg *et al.* 1987). These structural and compositional changes may affect the susceptibility of LDL fatty acids and cholesterol to oxidation, as was demonstrated for LDL subfractions isolated from healthy subjects (de Graaf *et al.* 1991). Decreased *in vitro* oxidizability of LDL by altering its composition in hypercholesterolaemic patients treated with simvastatin and pravastatin was also observed (Kleinveld *et al.* 1993).

In patients with familiar combined hyperlipidaemia, the prevalence of small, dense LDL particles with diminished oxidative resistance is one of the main factors that contribute to the increased risk of atherosclerosis (Dejager *et al.* 1993). Ciprofibrate has been shown to normalize the atherogenic LDL subspecies profile in patients with combined hyperlipidaemia, which resulted in a preferential reduction of the elevated levels of dense LDL subspecies (Bruckert *et al.* 1993, Chapman and Bruckert 1996). Therefore, the increased resistance of LDL to oxidation after ciprofibrate therapy observed in our study might be related to the ability of this drug to influence the LDL particle profile and ALP positively. We conclude that, despite the small number of patients studied, ciprofibrate produced a significant and favourable effect on LDL resistance to oxidation and this new observation implies the need for further study.

References

BUEGE J.A., AUST S.D.: Microsomal lipid peroxidation. *Meth. Enzymol.* **52**: 302–310, 1978.

BRUCKERT E., DEJAGER S., CHAPMAN M.J.: Ciprofibrate therapy normalises the atherogenic low-density lipoprotein subspecies profile in combined hyperlipidemia. *Atherosclerosis* **100**: 91–102, 1993.

CHAPMAN M.J., BRUCKERT E.: The atherogenic role of triglycerides and small, dense low density lipoproteins: impact of ciprofibrate therapy. *Atherosclerosis* **124** (Suppl): S21–S28, 1996.

DE GRAAF J., HAK-LEMMERS H.L.M., HECTORS M.P.C., DEMACKER P.N.M., HENDRIKS J.C.M., STALENHOEF A.F.H.: Enhanced susceptibility to *in vitro* oxidation of the dense low density lipoprotein subfraction in healthy subjects. *Arterioscl. Thromb.* **11**: 298–306, 1991.

DEJAGER S., BRUCKERT E., CHAPMAN M.J.: Dense low-density lipoprotein subspecies with diminished oxidative resistance predominate in combined hyperlipidemia. *J. Lipid. Res.* **34**: 295–308, 1993.

EL-SAADANI M., ESTERBAUER H., EL-SAYED M., GOHER M., NASSAR A.Y., JÖRGENS G.: A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. *J. Lipid. Res.* **30**: 627–630, 1989.

ESTERBAUER H., STRIEGL G., PUHL H., ROTHENEDER M.: Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Radic. Res. Commun.* **6**: 67–75, 1989.

GAW A., PACKARD C.J., CASLAKE M.J., GRIFFIN B.A., LINDSAY G.M., THOMPSON J., VALLANCE B.D., WOSORNU D., SHEPHERD J.: Effect of ciprofibrate on LDL metabolism in man. *Atherosclerosis* **108:** 137–148, 1994.

GOLDBERG A.C., SCHONFELD G., ANDERSON C., DILLINGHAM M.A.: Fenofibrate affects the composition of lipoproteins. *Am. J. Med.* **83** (Suppl. 53): 60–72, 1987.

HOFFMAN R., BROOK J.G., AVIRAM M.: Hypolipidemic therapy reduces lipoprotein susceptibility to undergo lipid peroxidation: in vitro and ex vivo studies. *Atherosclerosis* **93:** 105–113, 1992

HUSSEIN O., SCHLEZINGER S., ROSENBLAT M., KEIDAR S., AVIRAM M.: Reduced susceptibility of low density lipoprotein (LDL) to lipid peroxidation after fluvastatin therapy is associated with the hypocholesterolemic effect of the drug and its binding to the LDL. *Atherosclerosis* **128:** 11–18, 1997.

KLEINVELD H.A., HAK-LEMMERS H.L.M., STALENHOEF A.F.H., DEMACKER P.N.M.: Improved measurement of low density lipoprotein susceptibility to copper-induced oxidation; application of a short low-density lipoprotein isolation procedure. *Clin. Chem.* **38:** 2066–2072, 1992.

KLEINVELD H.A., DEMACKER P.N.M., DE HAAN A.F.J., STALENHOEF A.F.H.: Decreased in vitro oxidizability of low-density lipoprotein in hypercholesterolaemic patients treated with 3-hydroxy-3-metylglutaryl-CoA reductase inhibitors. *Eur. J. Clin. Invest.* **23:** 289–295, 1993.

LAVY A., BEN AMOTZ A., AVIRAM M.: Preferential inhibition of LDL oxidation by the all-trans isomer of β-carotene in comparison to the 9-cis-β-carotene. *Eur. J. Clin. Chem. Clin. Biochem.* **31:** 83–90, 1993.

LOWRY O.H., ROSEBROUGH N.J., FARR A.L., RANDALL R.J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275, 1951.

ORO L., CARLSON L.A., OLSSON A., POOLE P.H.: Long-term efficacy and safety of ciprofibrate in patients with primary hyperlipidemia. *Curr. Ther. Res.* **51:** 750–762, 1992.

PARTHASARATHY S., FONG L.G., OTERO D., STEINBERG D.: Recognition of solubilized apoproteins from delipidated, oxidized low density lipoprotein (LDL) by the acetyl-LDL receptor. *Proc. Natl. Acad. Sci. USA* **84:** 537–540, 1987.

SATO K., MIKI E., SHIMASAKI H.: Free radical-mediated chain oxidation of low density lipoprotein and its synergistic inhibition by vitamin E and vitamin C. *Arch. Biochem. Biophys.* **279:** 402–405, 1990.

STEINBERG D., PARTHASARATHY S., CAREW T.E., KHOO J.C., WITZTUM J.L.: Beyond cholesterol: modifications of low density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924, 1989.

STEINBRECKER U.P., PARTHASARATHY S., LEAKE D.S., WITZTUM J.L., STEINBERG D.: Modification of low density lipoproteins by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA* **81:** 3883–3887, 1984.

TERPSTRA A.H.M., WOODWARD C.J.H., SANCHEZ-MUNIZ F.J.: Improved techniques for the separation of serum lipoproteins by density gradient ultracentrifugation: visualization by prestaining and rapid separation of serum lipoproteins from small volumes of serum. *Anal. Biochem.* **111:** 149–157, 1981.

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