Gemfibrozil Metabolite Inhibits In Vitro Low-Density Lipoprotein (LDL) Oxidation and Diminishes Cytotoxicity Induced by Oxidized LDL

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We hypothesized that M1, a metabolite of gemfibrozil, may have antioxidant properties because of its hydroxylated phenol ring, 5-(4-hydroxy-2,5-dimethyl-phenoxy)-2,2-dimethyl pentanoic acid. The susceptibility of low-density lipoprotein (LDL) to oxidative modification was investigated by a method using 2,2-azobis(4-methoxy-2,4-dimethylvaleronitrile [MeO-AMVN]) or Cu^{2+} as previously reported. Conjugated dienes (CDs), lipid hydroperoxide (LPO), and thiobarbituric acid–reactive substances (TBARS) were measured to evaluate the degree of LDL oxidation. Oxidized LDL (OxLDL), which is used for cytotoxicity studies, was prepared by the dialysis method using Cu^{2+} as the oxidation inducer. Cytotoxicity induced by OxLDL was studied in J774 macrophages by colorimetric assay using 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT assay). The oxidative modification of LDL was inhibited by M1 in a dose-dependent manner. The antioxidant effect of M1 on LDL oxidation was diminished by dialysis of the LDL incubated with M1 against phosphate-buffered saline (PBS), suggesting that M1 is hydrophilic rather than lipophilic. M1 diminished the cytotoxicity induced by OxLDL, although it was milder versus probucol. These data suggest that this gemfibrozil metabolite has an antioxidant effect on LDL, and thus M1 may contribute to the antiatherogenic effects of gemfibrozil.

IT IS WELL ESTABLISHED that hypercholesterolemia is an important risk factor for coronary heart disease (CHD). Clinical intervention studies have confirmed the effectiveness of hypolipidemic therapy in reducing the sequelae of CHD.[1,2] The Helsinki Heart Study (HHS) further demonstrated that reducing total and low-density lipoprotein (LDL) cholesterol is effective in the primary prevention of CHD.[3] During the 5-year follow-up period of the HHS, cardiovascular events were reduced by 34% in the gemfibrozil-treated group. Since gemfibrozil decreases triglycerides in addition to decreasing LDL cholesterol, this may also be a component of its antiatherogenic effect.[4] Furthermore, a post hoc analysis of HHS findings indicates that an increase in high-density lipoprotein cholesterol is also a strong indicator of a reduction in future cardiovascular events.[4] Fujii and Sobel[5] have reported that the beneficial effects of gemfibrozil in reducing coronary events may also be a result of its potentiation of fibrinolysis by directly reducing the synthesis of endogenous plasminogen activator inhibitor type 1.

Qualitative changes in LDL, particularly its oxidation, have been considered to be very important for the formation of foam cells in vivo.[6,7] Oxidized LDL (OxLDL) is also toxic to a number of cell types, including macrophages, in vitro.[8,9] The acellular core of advanced atherosclerotic lesions contains dead macrophages, suggesting that the cytotoxic effects of OxLDL may contribute to the development of these lesions.[10-14] In addition, OxLDL has chemotactic properties and stimulates cytokine secretion, which may also contribute to the progression of atherosclerotic lesions.[7] In several clinical trials such as the Cambridge Heart Antioxidant Study (CHAOS), vitamin E supplementation was also suggested to prevent CHD in human subjects,[15] but the Heart Outcomes Prevention Evaluation (HOPE) study does not show a beneficial effect.[16]

Although the effect of vitamin E is controversial in the prevention of CHD, the most compelling evidence that oxidizedlipoproteins are involved in atherogenesis is that the development of atherosclerosis can be markedly attenuated in experimental animals by treatment with antioxidants.[17] M1, a metabolite of gemfibrozil with a hydroxylated phenol ring, 5-(4-hydroxy-2,5-dimethyl-phenoxy)-2,2-dimethyl pentanoic acid (Fig 1), may have antioxidant properties, as we reported previously for structurally similar compounds.[18] In a preliminary study, we demonstrated that M1 inhibited in vitro oxidation of LDL induced by cupric ion or a new azo compound, 2,2-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN).[19] In the present study, we have further defined the antioxidant effect of M1 and its effects on the OxLDL-induced cytotoxicity of macrophages.

MATERIALS AND METHODS

Materials

RPMI 1640 medium and MeO-AMVN were purchased from GIBCO (Grand Island, NY) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Gemfibrozil and M1 were supplied by Warner-Lambert K.K. Japan (Tokyo, Japan). Probucol was supplied by Daiichi Pharmaceutical (Tokyo, Japan). All other chemicals were obtained from Sigma Chemical (St Louis, MO).

Kinetics of Azo Compound (MeO-AMVN)- and Cu^{2+}-Mediated LDL Oxidation

LDL (d = 1.019 to 1.063 g/mL) was separated from normal control plasma by ultracentrifugation using a vertical rotor (RP67-VF; Hitachi, Tokyo, Japan) according to the method of Chung et al.[20] Centrifugation was performed at 65,000 rpm for 90 minutes at 4°C. The kinetics of MeO-AMVN- and Cu^{2+}-mediated LDL oxidation was measured by determining the change in absorbance at 234 nm on a Shimazu UV-160A spectrophotometer (Kyoto, Japan) with 6 cuvette positions according to the method of Kondo et al[21] and Esterbauer et al.[22] After isolation, the oxidation of LDL (100 μg/mL) mediated by MeO-AMVN (final concentration, 400 mmol/L) without dialysis against phosphate-buffered saline ([PBS] pH 7.4) was immediately and continuously

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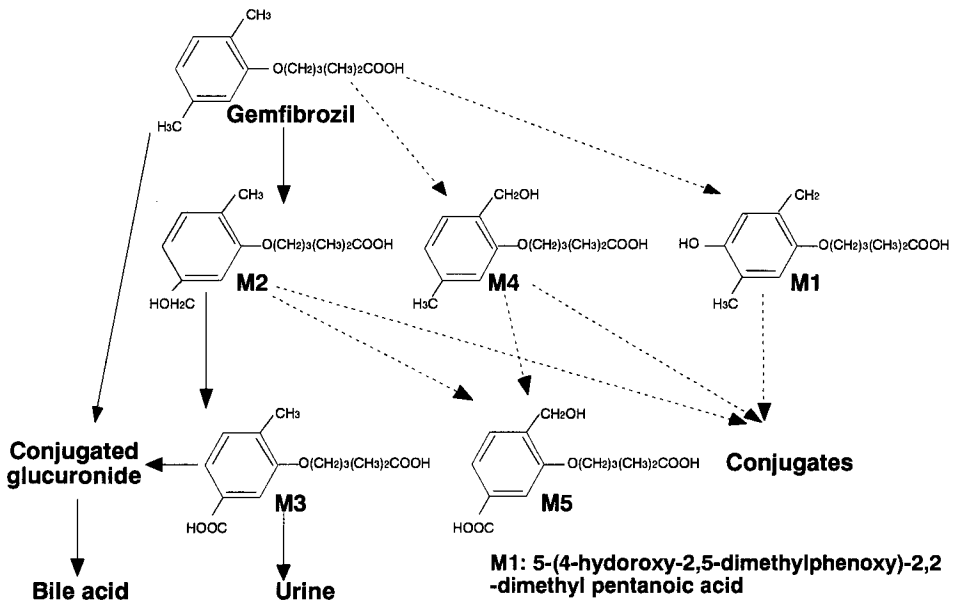

Fig 1. The metabolic pathway of gemfibrozil in the rat.

monitored at 37°C for 5 hours in the absence or presence of the various reagents already described, because MeO-AMVN–mediated oxidation is not affected by EDTA.[21] Since MeO-AMVN is a lipid-soluble azo compound and is thermally decomposed to produce peroxyl radicals at constant rates within the lipid phase, it can induce the oxidation of LDL without interference with the absorbance of conjugated dienes (CDs) at 234 nm.[21] However, the LDL for use in Cu^{2+}-mediated LDL oxidation was dialyzed for 20 hours against PBS at 4°C.[22] The oxidation of LDL (150 μg/mL) mediated by Cu^{2+} (final concentration, 5 μmol/L) was monitored by the MeO-AMVN method. To determine whether an antioxidant, particularly M1, is hydrophilic or lipophilic, the kinetics of oxidation of LDL mediated by MeO-AMVN was monitored after LDL was incubated with various antioxidants at 4°C for 30 minutes (0 hours) and then dialyzed at 4°C for 6 and 20 hours against PBS containing 0.5 mmol/L EDTA.[23-25]

Extent of Modification of OxLDL Mediated by Cu^{2+}

Cu^{2+}-mediated oxidation was stopped by refrigeration (4°C) and the addition of both diethylene triamine pentaacetic acid (final concentration, 50 μmol/L) and butylated hydroxytoluene (final concentration, 25 μmol/L).[26,27] The formation of CD in LDL was measured essentially by its absorbance at 234 nm. Lipid peroxides (LPO) in LDL were measured according to the method of el-Saasani et al.[28] Malondialdehyde (MDA) and MDA-like compounds reacting with thiobarbituric acid (TBA) were measured according to the method of Slater,[29] with slight modifications. Compounds that react with TBA (TBARS) were measured spectrophotometrically at 535 nm.

Cytotoxicity of Macrophages

OxLDL for cytotoxicity studies was prepared by dialyzing the LDL against at least 100 vol dialysate including 30 μmol/L $CuSO_4$[30] at 4°C for 16 hours. This method does not require diluted LDL for oxidation. However, another buffer (0.01 mol/L Tris and 0.15 mol/L NaCl, pH 7.4) is used because $CuSO_4$ forms the precipitate in PBS at the low temperature. To stop oxidation of the LDL, 0.5 mmol/L EDTA was added, and the OxLDL was stored at 4°C until cytotoxicity studies.[23-25] We have found TBARS levels of 16 to 30 nmol MDA/mg LDL protein and LPO levels of 230 to 500 nmol/mg LDL protein. For the cytotoxicity studies, J774 macrophages were used. The cells were seeded at a density of 0.5×10^6/mL in 96-well plates in medium containing 5% (vol/vol) lipoprotein-deficient fetal bovine serum.

The cytotoxicity of OxLDL was determined by the MTT assay according to the method of Mosmann.[31,32] This assay is based on the ability of cells to reduce the yellow, water-soluble dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to a blue, insoluble formazan product. At the indicated times, stock MTT solution (15 μL per 150 μL medium) was added to all wells of an assay and the plates were incubated at 37°C for 3 hours. Dimethyl sulfoxide was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After 25 minutes at 37°C in 5% CO_2, the plates were read on a micro–enzyme-linked immunosorbent assay reader (MPR-A4i; Tosoh, Tokyo, Japan). Cytotoxicity is expressed relative to the cell viability of a lipoprotein-free well. According to this method, the coefficient of variation for cytotoxicity induced by native LDL, OxLDL, and OxLDLs including gemfibrozil, M1, and probucol was 12.1%, 34.0%, 25.2%, 8.8%, and 4.3%, respectively.

Protein concentration was measured by the method of Lowry et al[33] using bovine serum albumin as a standard.

Statistical Analysis

Results are expressed as the mean ± SE. All data were analyzed by Student's unpaired 2-tailed *t* test.

RESULTS

Effect of M1 and Probucol on Azo Compound- and Cu^{2+}-Mediated LDL Oxidation

Figure 2 shows the kinetic curves of CD produced by MeO-AMVN- and Cu^{2+}-mediated LDL oxidation and the effect of M1 and probucol on these curves. M1 and probucol inhibited the oxidation of LDL in a dose-dependent manner. Probucol at a concentration of 10 μmol/L abolished the oxidative modification of LDL by both MeO-AMVN and Cu^{2+}, but a similar concentration of M1 incompletely inhibited oxidation, suggesting that M1 has a weaker effect than probucol in inhibiting the oxidation of LDL.

To determine whether M1 is hydrophilic or lipophilic, the kinetics of LDL oxidation mediated by MeO-AMVN was

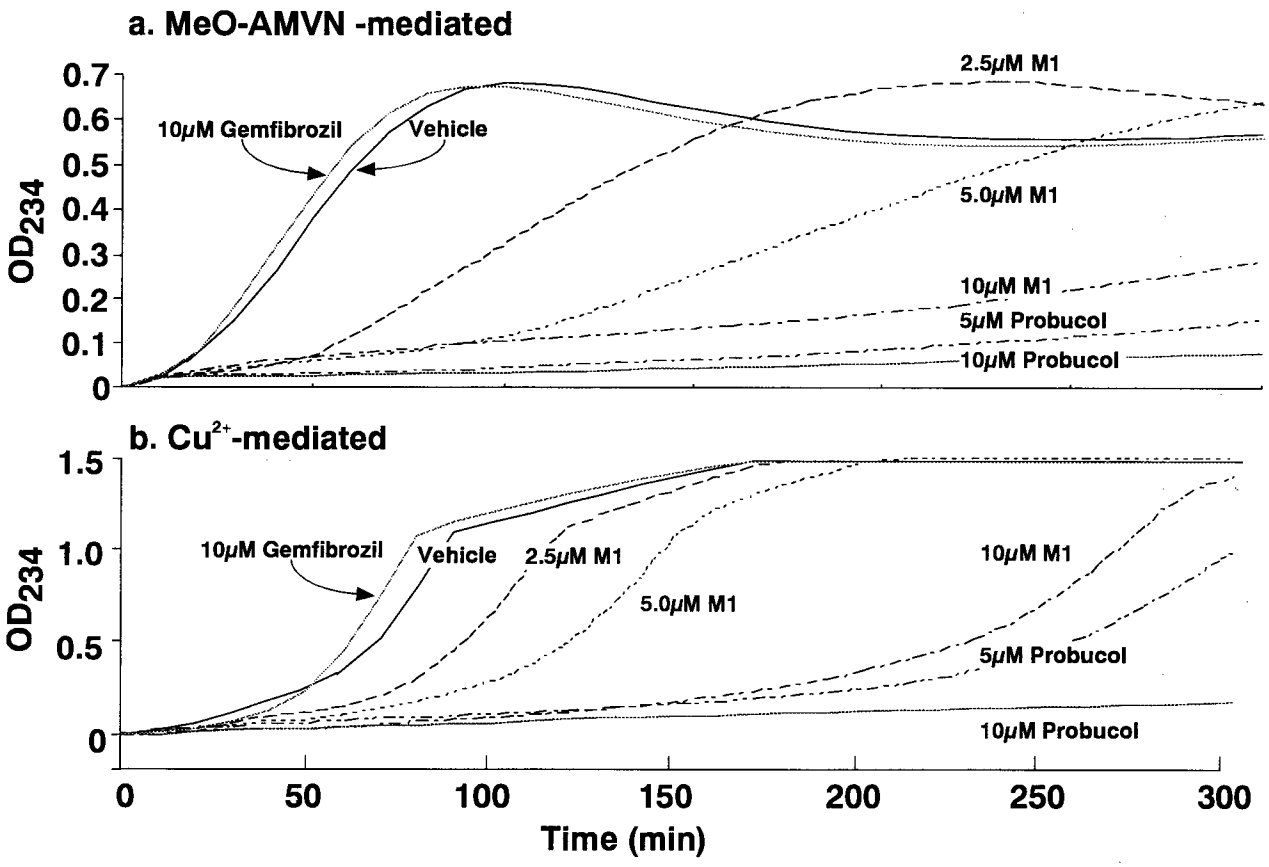

Fig 2. Effects of M1 and probucol on LDL oxidation mediated by an azo compound and Cu^{2+}. (a) An LDL protein concentration of 100 μg/mL and a MeO-AMVN concentration of 400 μmol/mL were used. (b) An LDL protein concentration of 150 μg/mL and a Cu^{2+} concentration of 5 μmol/L were used. Reactions were monitored as described in the Methods except that M1 (2.5, 5, or 10 μmol/L final concentration) or probucol (5 or 10 μmol/L final concentration) was added to the reaction mixture. Data are typical of 5 experiments.

monitored at 37°C for 5 hours after LDL (100 μg/mL) incubation with various antioxidants and dialysis against PBS at 4°C for 6 and 20 hours. Vitamin C was used as a positive control for the hydrophilic antioxidants, and vitamin E and probucol as a positive control for the lipophilic antioxidants. M1 (10 μmol/L) had a similar antioxidant effect on MeO-AMVN-mediated LDL oxidation, and its antioxidant effects were weaker than 40 μmol/L vitamin C or 10 μmol/L probucol but stronger than 10 μmol/L vitamin E (Fig 3a). Figure 3b and c shows the effects of 6 and 20 hours of dialysis of LDL, incubated with various antioxidants, on the extent of oxidative modification of the LDL. Although vitamin C lost its antioxidant effect completely after 6 hours of dialysis, M1 did not. The antioxidant effect of M1 was still stronger than that of vitamin E. After 20 hours of dialysis, both vitamin C and M1 lost their antioxidant effects (Fig 3c). On the other hand, vitamin E and probucol did not lose their antioxidant effects even after 6 or 20 hours of dialysis (Fig 3b and c). Gemfibrozil itself did not affect the oxidative modification of LDL.

Inhibition of Cu^{2+}-Mediated LDL Oxidation by M1 and Probucol

To confirm the antioxidant effects of M1 and probucol, we measured the parameters of Cu^{2+}-mediated oxidation. M1 significantly inhibited the formation of CD as compared with the control, although its inhibitory effect was milder than that of probucol (Fig 4a). M1 and probucol significantly inhibited the formation of LPO and TBARS (Fig 4b and c). Again, gemfibrozil itself did not inhibit LDL oxidation.

Effect of M1 and Probucol on Cytotoxicity Induced by OxLDL

Pilot experiments to determine the optimal OxLDL dose and incubation time were performed. The cytotoxicity of OxLDL, but not native LDL, on J774 macrophages after a 48-hour incubation was dose-dependent. Probucol inhibited the cytotoxic effects of OxLDL significantly. M1 also inhibited these cytotoxic effects, although the effects of M1 were weaker than those of probucol. Gemfibrozil did not inhibit cytotoxicity induced by OxLDL (data not shown). Figure 5 shows that M1 and probucol inhibited the cytotoxic effects of OxLDL significantly.

DISCUSSION

We previously reported that M1 possesses antioxidant effects.[17] Aviram et al[34] examined these in more detail. In the present study, we have further investigated not only the antioxidant effects of M1 on LDL oxidation but also the protective effects against cytotoxicity induced by OxLDL.

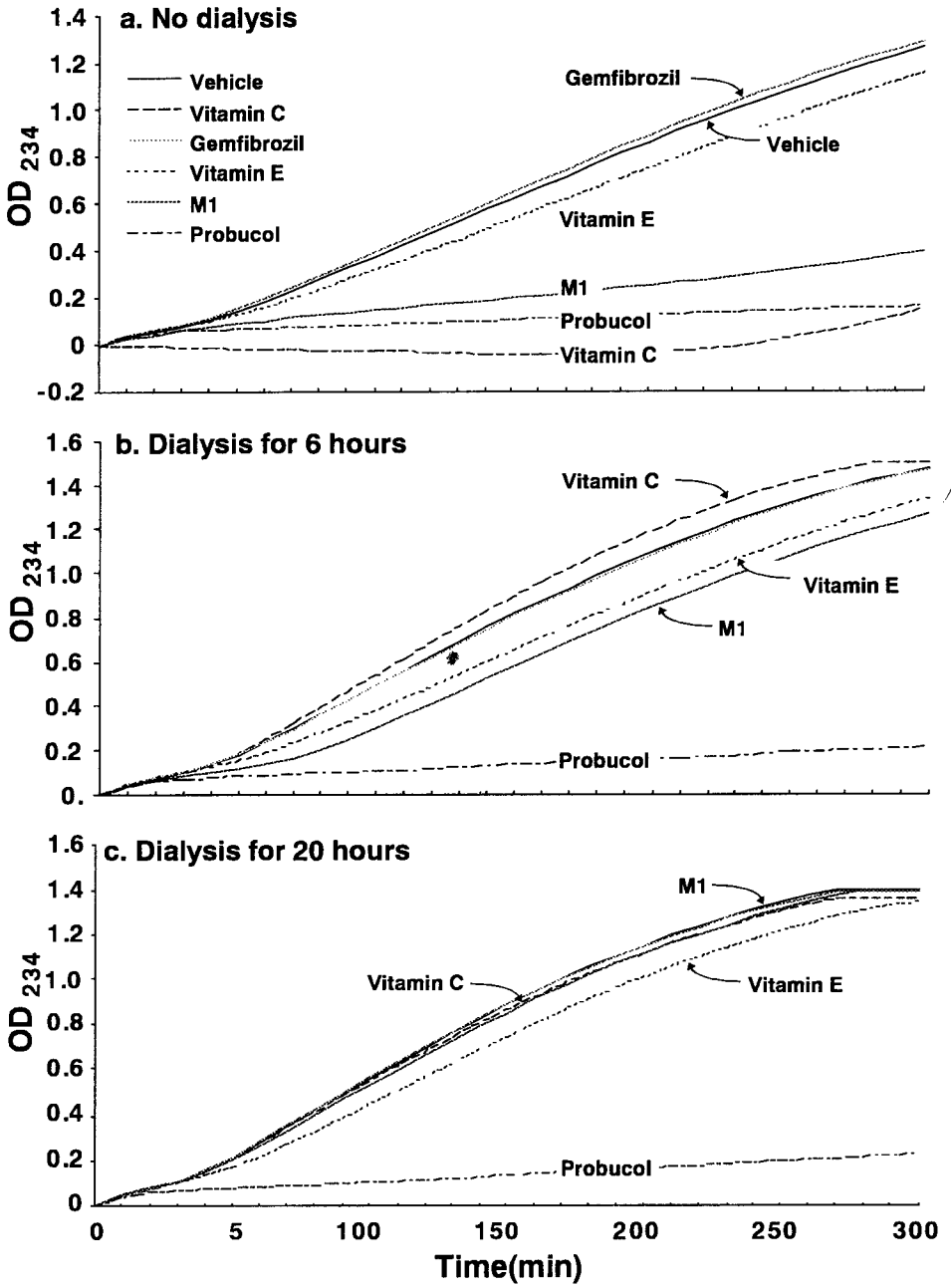

Fig 3. Effects of dialysis on LDL incubated with antioxidants on the formation of conjugated diene. (a) An LDL protein concentration of 100 μg/mL and a MeO-AMVN concentration of 400 μmol/L were used. Concentrations of other compounds, except vitamin C, were 10 μmol/L. A vitamin C concentration of 40 μmol/L was used. (a) The antioxidant effect on MeO-AMVN–mediated LDL oxidation at 0 hours. (b) the antioxidant effect after 6 hours of dialysis against PBS. (c) The antioxidant effect after 20 hours of dialysis against PBS. Data are typical of 5 experiments.

When a single dose of 1,350 mg gemfibrozil was orally administered to 6 healthy subjects, plasma concentrations of M1 were maximal, 8.7 ± 5.5 μmol/L (mean ± SD), after 4 hours. Based on this information, the M1 concentrations used in this study were between 2.5 and 10 μmol/L.

M1 inhibited both Cu^{2+}- and MeO-AMVN-mediated oxidation of LDL, indicating that it does not act merely as a chelater of Cu^{2+}.[23-25] To learn whether M1 is hydrophilic or lipophilic, we compared the effects of dialyzing LDL incubated with M1 on oxidation by MeO-AMVN versus those from vitamin C,[35] vitamin E,[36] and probucol.[37] Although the antioxidant effect of vitamin C on the lipid peroxidation of LDL was abolished by 6 hours of dialysis against PBS, the effect of M1 was diminished only slightly by 6 hours and abolished completely by 20 hours of dialysis. Vitamin E and probucol maintained their effects even after 20 hours of dialysis. These data suggest that M1 is more lipophilic than vitamin C but more hydrophilic than probucol or vitamin E, and should bind to LDL loosely. Thus, M1 may protect LDL from oxidative modification by binding to it.

Negre-Salvayre et al[38] have reported that the cytotoxicity of LDL treated with UV light in the presence of antioxidants (protected UV-treated LDL) correlated well with its content of lipid peroxidation markers such as CD, TBARS, and fluorescent lipid-soluble products. We have confirmed the effects of probucol and M1 on the production of CD, LPO, and TBARS in OxLDL. Furthermore, we investigated the direct effect of these antioxidants on the cytotoxicity of OxLDL. M1 and probucol

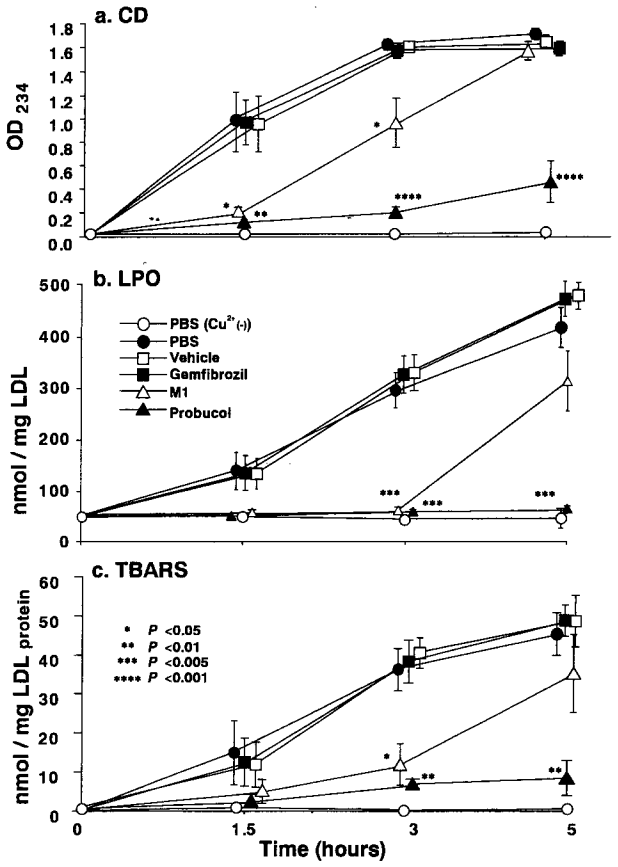

Fig 4. Inhibition of Cu^{2+}-mediated LDL oxidation by M1 and probucol. An LDL protein concentration of 150 μg/mL and a Cu^{2+} concentration of 5 μmol/L were used. Reactions were stopped by the addition of both diethylene triamine pentaacetic acid (final concentration, 50 μmol/L) and butylated hydroxytouluene (final concentration, 25 μmol/L) at the indicated times, as described in the Methods. Results are expressed as the mean ± SE (n = 5). *$P < .05$ v control, **$P < .01$ v control, ***$P < .005$ v control, ****$P < .001$ v control.

not only inhibit LDL oxidation but also protect against cytotoxicity induced by OxLDL.

During the 5-year follow-up period of the HHS, gemfibrozil reduced cardiovascular events by 34% even though the reduction of cholesterol was only 8%.[4] The Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT) suggested that a 1% reduction in cholesterol induces a 2% reduction in cardiac events.[1,2] Gemfibrozil appears more effective in preventing cardiac events than the cholestyramine used in the LRC-CPPT, suggesting that gemfibrozil possesses effects other than simply a reduction of serum cholesterol.[3] The primary actions of gemfibrozil are to decrease LDL cholesterol and triglycerides[4] and increase high-density lipoprotein cholesterol. However, it has been suggested recently that gemfibrozil may have antiatherogenic effects by changing the composition of LDL and very–low-density lipoprotein,[39] increasing the particle size and decreasing the density of LDL,[40] and decreasing insulin resistance[41] and the synthesis of endogenous plasminogen activator inhibitor type 1.[5] Since the size of LDL is critical for its oxidative modification, gemfibrozil may protect against the oxidative modification of LDL by increasing its particle size.

Recent data that demonstrate the presence of oxidized lipoproteins in vivo, combined with studies showing a protective effect of antioxidants against atherosclerosis, support the theory that lipoprotein oxidation is causally related to arterial disease.[42-44] In conclusion, the present study shows that this gemfibrozil metabolite (M1) has antioxidant effects on LDL oxidation and protective effects against cytotoxicity induced by OxLDL, which may contribute to the antiatherogenic effects of gemfibrozil.

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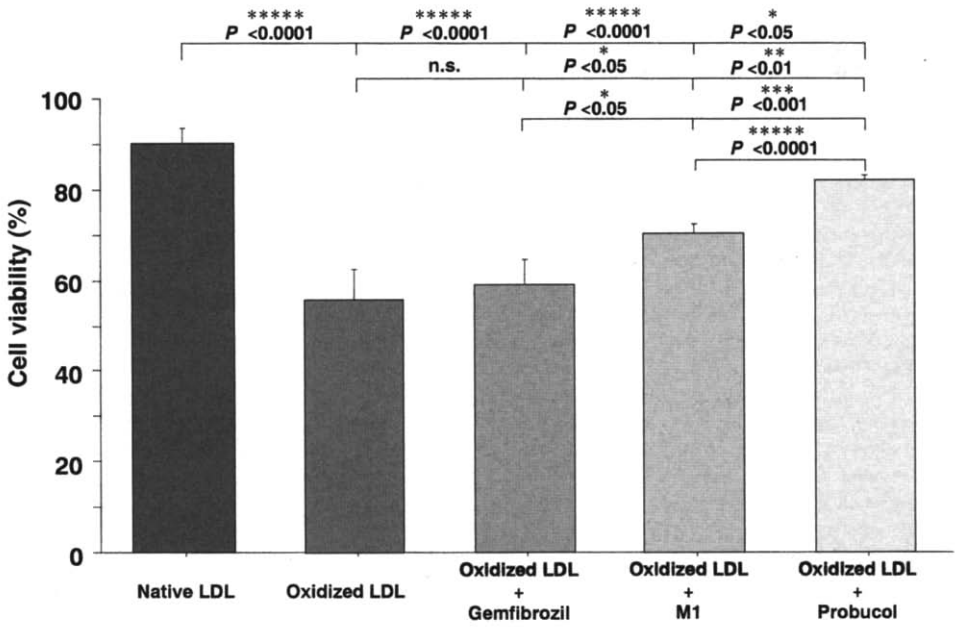

Fig 5. Effect of antioxidants on cytotoxicity induced by oxidized LDL. Cytotoxicity studies were performed on J774 macrophages. Cell viability was evaluated after cells were incubated for 48 hours. Results are expressed as the mean ± SE (n = 8); ns, not significant.

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