Probucol prevents oxidative injury to endothelial cells

Masafumi Kuzuya, Michitaka Naito, Chiaki Funaki, Toshio Hayashi, Kanichi Asai, and Fumio Kuzuya
Department of Geriatrics, Nagoya University School of Medicine, Nagoya 466, Japan

Abstract We evaluated the effect of the antioxidant, probucol, on the cytotoxic effects of oxidized low density lipoprotein (OX-LDL) or of cumene hydroperoxide (CumOOH) on cultured bovine endothelial cells (EC). The addition of CumOOH to EC caused the release of lactate dehydrogenase and the accumulation of thiobarbituric acid-reacting substances (TBARS), effects that were protected against by preincubation with either probucol or tocopherol. Similarly, preincubation of EC with those antioxidants protected against OX-LDL toxicity and the accumulation of TBARS. The content of probucol in EC measured by high performance liquid chromatography was directly correlated with the extent of protection against OX-LDL toxicity. We also found that treatment of EC with serum from patients receiving treatment with probucol resulted in the detection of probucol in the cells. We conclude that probucol is transported and incorporated into EC membranes to act as a radical-trapping antioxidant, protecting the EC against oxidative stress. Our results also indicate that lipid peroxidation in cellular membranes involves cell injury inflicted by OX-LDL.-Kuzuya, M., M. Naito, C. Funaki, T. Hayashi, K. Asai, and F. Kuzuya. Probucol prevents oxidative injury to endothelial cells. J. Lipid Res. 1991. 32: 197-204.

Supplementary key words lipid peroxidation • cumene hydroperoxide • oxidized low density lipoprotein • dl-α-tocopherol • atherosclerosis • cytotoxicity

Recent studies suggest that the oxidative modification of low density lipoprotein (LDL) may play an important role in the pathogenesis of atherosclerosis. In vitro studies of cellular interactions with LDL have revealed that monocytes (1) and vascular cells such as endothelial cells (EC) (2, 3) and smooth muscle cells (3, 4) can modify LDL by peroxidation. That modification then leads to functional changes in LDL properties as LDL uptake and degradation by macrophages through a scavenger receptor (5, 6), cytotoxicity to cultured EC (7), and increased chemotactic properties in monocytes (8). These functional changes induced in LDL by peroxidation would be relevant to atherogenesis.

Previous studies have shown that probucol, a drug widely used in the treatment of hypercholesterolemia, blocks the cell-mediated and copper-promoted oxidation of LDL through its antioxidant properties (9). Recently Kita et al. (10) and Carew, Schwenke, and Steinberg (11) reported that probucol prevents the progression of atherosclerosis in the Watanabe Heritable Hyperlipidemic rabbit (WHHL rabbit).

We investigated the effect of probucol on oxidized LDL (OX-LDL)-induced damage to cultured EC, and free radical-mediated cellular damage induced by cumene hydroperoxide (CumOOH). The latter is an organic peroxide known to initiate lipid peroxidation (12-14).

MATERIALS AND METHODS

Materials
Probucol was kindly supplied by Merrell Dow Pharmaceutical Co. (USA) and dl-α-tocopherol (tocopherol) was supplied by Eizai Pharmaceutical Co. (Japan). Trolox C was kindly supplied by Dr. Takao Kaneko of the Tokyo Metropolitan Institute of Gerontology. Butylated hydroxytoluene (BHT) and cumene hydroperoxide (CumOOH) were purchased from Sigma Chemical Co. (USA) and thiobarbituric acid (TBA) was from Wako (Japan). Dulbecco's modified Eagle's medium (DME), phenol red-free Eagle's minimum essential medium (MEM), and Dulbecco's modified Eagle's medium (DME), phenol red-free Eagle's minimum essential medium (MEM), and Dulbecco's phosphate-buffered saline (PBS) were obtained from Nissui Pharmaceutical Co. (Japan). Bovine calf serum (CS) was obtained from Cell Culture Laboratories (USA). Flasks and 16-mm 24-well plates were obtained from Corning Glassworks (USA).

Abbreviations: EC, endothelial cells; LDL, low density lipoprotein; OX-LDL, oxidized low density lipoprotein; LPDS, lipoprotein-deficient serum; TBA(RS), thiobarbituric acid (reacting substances); DME, Dulbecco's modified Eagle's medium; MEM, Eagle's minimum essential medium; CS, calf serum; PBS, Dulbecco's phosphate-buffered saline; LDH, lactate dehydrogenase; CumOOH, cumene hydroperoxide; TCA, trichloroacetic acid; MDA, malondialdehyde; HPLC, high performance liquid chromatography.
Cell culture

Cultured EC were established from the thoracic aorta of the fetal calf as previously described (15) and maintained in DME supplemented with 10% (by volume) CS, penicillin, streptomycin, and amphoteracin in a humidified atmosphere containing 5% CO2 and 95% air. Cells at passage 6–13 were used for all experiments.

Preparation of oxidized low density lipoprotein

LDL (d 1.019–1.063 g/ml) was isolated from normal human plasma by ultracentrifugation as described previously (16). The LDL preparation was dialyzed at 4°C for 48 h against four changes of at least 50 volumes of 0.15 M NaCl. The LDL preparation was dialyzed at 4°C for 48 h against four changes of at least 50 volumes of dialyzate (0.15 M NaCl, pH 7.4). OX-LDL was prepared through additional dialysis for 48 h against four changes of at least 50 volumes of dialyzate (5 µM FeSO4 in 0.15 M NaCl, pH 198). OX-LDL was dialyzed at 4°C for 48 h against four changes of at least 100 volumes of dialyzate (0.15 M NaCl in distilled water). Lipoprotein-deficient serum (LPDS, d > 1.25 g/ml) was prepared by ultracentrifugation of CS and dialyzed at 4°C for 48 h against 0.15 M NaCl.

Assessment of injury to EC

Cellular injury was assessed by measuring the amount of lactate dehydrogenase (LDH) released from the cells into the medium. EC at confluence in 24-well plates (5.2 × 10⁵ cells/well) were rinsed with PBS and incubated in a total volume of 0.5 ml of MEM containing various concentrations of CumOOH or OX-LDL. In some trials, MEM containing CumOOH or OX-LDL supplemented with probucol, tocopherol, or Trolox C was used as the experimental medium. CumOOH was diluted with MEM to the desired final concentration. OX-LDL preparations were diluted with saline before use so that the desired final concentrations of each fraction could be attained in the culture medium with the 30% medium dilution. Probucol or tocopherol was first dissolved in 99.5% ethanol (10 mM) and added to the medium at a final concentration of 50 µM (0.5% ethanol by volume). Trolox C in stock solution (10 mM in MEM) was added to the medium at a final concentration of 25 µM or 50 µM. After incubation for the indicated times at 37°C, the medium was removed and LDH activity was determined by spectrophotometric analysis of NADH oxidation (Shimadzu spectrophotometer UV-160) (17). Each LDH activity was compared with the LDH released from cells after the addition of Triton X-100 for 0.1% final concentration (% total LDH release).

Pretreatment with probucol or tocopherol

Confluent endothelial monolayers in 24-well plates were incubated in DME containing 10% CS supplemented with probucol (probucol-treated cells) or with tocopherol (tocopherol-treated cells) at 37°C for 16 h except where noted. Cells were then rinsed 3 times with PBS and used for the assay of cellular injury. The antioxidant was first dissolved in 99.5% ethanol (10 mM), then added to DME containing 10% CS at a final concentration of 50 µM (0.5% ethanol by volume). Control cultures were treated with DME containing 10% CS with the same amount of ethanol.

Measurement of lipid peroxide levels

The formation of thiobarbituric acid-reacting substances (TBARS) was used as an indicator of lipid peroxidation. Confluent EC in T-25 flasks (4.5 × 10⁶ cells/flask) were incubated at 37°C in 2 ml of MEM containing various concentrations of CumOOH or OX-LDL for the indicated times. The OX-LDL preparation was diluted with saline and added to MEM at 30% by volume. Cells were then scraped off with a rubber spatula, centrifuged for 5 min at 400 g, and washed twice with PBS. The cells were resuspended in 0.6 ml of PBS, and a 0.5-ml aliquot of the cell suspension was analyzed for protein content according to the method of Lowry et al. (19). To determine TBARS in the LDL preparation, 100 µl of LDL was mixed with 1.5 ml of 20% TCA and 1.5 ml of 0.67% TBA. The mixture was incubated at 97° for 20 min, cooled, and centrifuged. The supernatant fraction was assayed fluorometrically. In these studies malondialdehyde (MDA) formed from 1,1,3,3-tetramethoxypropane was used as a standard.

Determination of intracellular probucol content

Probucol content in cells was assayed using a modification of the high performance liquid chromatography (HPLC) procedure described previously (20). EC at confluence in T-25 flasks (4.5 × 10⁶ cells/flask) were treated with DME containing 10% CS supplemented with probucol (50 µM) for the indicated times. In some cases, EC were treated with DME containing 9.5% LPDS (protein concentration in the medium was coincident with 10% CS) supplemented with probucol (50 µM) or DME containing 20% serum from patients who were receiving probucol. Cells were rinsed with PBS to remove extracellular probucol, harvested with 0.1% trypsin/EDTA, and centrifuged at 400 g for 10 min. Cells were washed twice with PBS, suspended in 1 ml of PBS, and sonicated at 40 watts for 10 sec using an ultrasonic disrupter (Model UR-200P, Tomy Seiko Japan). To 200 µl of sample, 3 ml of ethanol (99.5%) was added. The mixture was shaken vigorously and centrifuged at 1700 g for 10 min. A portion of the supernatant was transferred to a centrifuge tube and evaporated under a stream of nitrogen. The residue was dissolved in 1.0 ml of 0.2 N NaOH and 5.0 ml of diethyl
ether. The mixture was shaken and centrifuged. The ether layer (4.5 ml) was evaporated to dryness under nitrogen. The residue was dissolved in 200 μl of methanol and an aliquot (40 μl) was analyzed by HPLC (Hitachi 655) on a Nucleosil 5C18 column (4.6 × 250 mm) with 3% acetic acid-acetonitrile 15:85 (v/v) as mobile phase at a flow rate of 2.0 ml/min. A Shimadzu SPD-6A was used as the UV detector (at 254 nm) and peak areas were quantitated with a Shimadzu Data Processor Chromatopac C-R4A. The concentration of probucol was quantitated from a standard curve constructed from samples to which were added specified concentrations of probucol (1, 5, and 10 μg/ml) prior to extraction of the sample. The lower detection limits for these standard curves was 0.2 μg probucol/ml.

Other assays
Cholesterol was determined by the established procedure (21). Agarose gel electrophoresis of LDL was carried out as described by Noble (22). Data were expressed as means ± standard deviation (SD); statistical significance was determined by Student’s t test. Probability level was P<0.05.

RESULTS

Effect of probucol and tocopherol on CumOOH toxicity
MEM containing CumOOH caused a dose-related release of LDH from EC into the medium (Fig. 1A), and induced a significant increase in the formation of TBARS in the cells (Table 1). Supplementation of 50 μM of probucol or tocopherol to MEM containing CumOOH either had no effect (probucol) or a limited effect (tocopherol) on the release of LDH induced by CumOOH (Fig. 1A). When the cells were preincubated for 16 h with DME containing 10% CS supplemented with 50 μM of probucol or tocopherol, rinsed with PBS, and then exposed to MEM containing CumOOH, the release of LDH was significantly reduced as compared with control (Fig. 1B). In addition, the formation of TBARS in either the probucol- or tocopherol-treated cells after 4 h of incubation with MEM containing CumOOH was significantly less than control (Table 1).

Effect of probucol and tocopherol on OX-LDL toxicity
Dialysis of the LDL preparation with a dialyzate containing 5 μM FeSO₄ led to a significant increase in the content of TBARS (OX-LDL, 8.2–13.4 nmol MDA/mg cholesterol; native LDL, 0.11 nmol MDA/mg cholesterol) and a marked increase in the electrophoretic mobility of the LDL preparation (Fig. 2), indicating the lipid per-oxidation of LDL and an increased negative charge of the LDL particle. With OX-LDL from 350 to 700 μg cholesterol/ml, 8 h of incubation with EC caused a marked release of LDH from EC (Fig. 3A). Similarly with CumOOH, supplementation of 50 μM of probucol or tocopherol to MEM containing OX-LDL could not protect against OX-LDL toxicity (Fig. 3A). To exclude the possibility of the involvement of radicals in the aqueous phase in the toxicity of OX-LDL, we also ex-
TABLE 1. Thiobarbituric acid-reacting substances (TBARS) in cells incubated with cumene hydroperoxide (CumOOH)

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Control Cells</th>
<th>Tocopherol-Treated Cells</th>
<th>Probucol-Treated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.47 ± 0.10</td>
<td>0.45 ± 0.08</td>
<td>0.45 ± 0.05</td>
</tr>
<tr>
<td>CumOOH, 2 mM</td>
<td>1.82 ± 0.14</td>
<td>1.21 ± 0.05*</td>
<td>1.27 ± 0.17*</td>
</tr>
<tr>
<td>CumOOH, 5 mM</td>
<td>2.65 ± 0.22</td>
<td>1.44 ± 0.25*</td>
<td>1.51 ± 0.11*</td>
</tr>
</tbody>
</table>

EC at confluence in T-25 flasks were incubated with MEM containing CumOOH for 4 h at 37°C. Then TBARS in cells were determined as described in Materials and Methods. Values represent the means ± SD of triplicate flasks.

*P < 0.01; †P < 0.05 versus control cells incubated with CumOOH 2 mM.

'P < 0.01 versus control cells incubated with CumOOH 5 mM.

Amined the effect of a water-soluble tocopherol analog, Trolox C. Supplementation of Trolox C (25 or 50 μM) protected EC against OX-LDL toxicity by only 4.5% or 8% (Fig. 4).

However, when cells were pretreated with DME containing 10% CS supplemented with 50 μM of probucol or tocopherol for 16 h and rinsed with PBS, there was a dramatic inhibition of LDH release (Fig. 3B). As shown in Fig. 5A, the degree of protection against OX-LDL was dependent on the duration of preincubation with probucol. To assess the accumulation of TBARS in the cells incubated with OX-LDL, confluent EC in T-25 flasks were exposed to 300 μg or 600 μg cholesterol/ml of OX-LDL. The large amounts of TBARS were found in the cells after 8 h incubation with OX-LDL (Table 2). When the cells were pretreated with probucol (50 μM) or tocopherol (50 μM) for 16 h, the accumulation of TBARS in the cells after incubation with OX-LDL was significantly reduced as compared with control cells (Table 2).

**Proabul content in EC**

The content of probucol in EC incubated with DME containing 10% CS supplemented with 50 μM probucol was detected by HPLC. After 24 h incubation with 50 μM of probucol, 0.69 μg probucol (approximately 1.3 μM)/10^6 cells was detected. Fig. 5B illustrates the probucol content in the EC treated with this agent for the intervals shown. The amount of probucol in the cells increased during incubation with this agent and was correlated with the degree of protection against OX-LDL toxicity (Fig. 5A, B). When EC were incubated for 24 h with DME containing 9.5% LPDS plus 50 μM probucol, the probucol content detected in cells was as much as that in the cells that were incubated with DME containing 10% CS plus 50 μM probucol (data not shown). We also examined the probucol content in EC that were treated with serum from patients receiving treatment with probucol, 500 mg twice daily. The levels of probucol in the serum of the two patients were 35.1 and 64.4 μM. After incubation of EC with DME supplemented with 20% (v/v) serum from the patients for 72 h, the corresponding probucol content in EC was 0.4 and 0.6 μM/3.8 x 10^6 cells.

**DISCUSSION**

Recently, attention has focused on such lipid peroxides as OX-LDL in the initiation and progression of atherosclerosis. The oxidation of LDL converts it to a form that is recognized by the scavenger receptor of macrophages, therefore it could potentially contribute to the formation of foam cells (5, 6). Recent evidence for the potential importance of OX-LDL is provided by in vivo studies showing the reduced formation of arterial lesions in atherosclerosis-prone rabbits (WHHL rabbits) treated with probucol (10, 11). One of the mechanisms of the antiatherogenic effect of probucol has been considered to be its inhibition of the oxidative modification of LDL, and thus, inhibition of foam cell formation (10, 11). Peroxidation of

Fig. 2. Agarose electrophoresis of native LDL and oxidized LDL.
 LDL in the arterial wall may contribute to atherogenesis in other ways. For example, OX-LDL is toxic to cultured EC (7), and endothelial damage caused by OX-LDL may play an important role in the initiation of the atherosclerotic process.

In this study we investigated the effect of probucol and another lipid-soluble antioxidant, tocopherol, on the injury induced by OX-LDL or by an organic peroxide, CumOOH, which is a suitable compound for studying the effects of lipid hydroperoxides. CumOOH, a substrate for glutathione peroxidase (23), is easily taken up by cells and rapidly provokes lipid peroxidation and damage to various organs and cells (12–14). We found that CumOOH caused a dose-related injury to EC as evidenced by a dose-dependent increase in the release of LDH from EC. Measurements of TBARS content in cells showed the formation of lipid peroxides following CumOOH treatment.

When EC were pretreated with either probucol or tocopherol, the release of LDH from cells especially treated with 2 mM CumOOH was significantly attenuated. Pretreatment with these antioxidants also significantly reduced the formation of TBARS in cells. Tocopherol is known to be transported and incorporated into the membranes and to inhibit free radical chain involved in the peroxidation of membrane lipids (24). The protective effects of preincubation with tocopherol on cellular injury and on the accumulation of lipid peroxides induced by CumOOH indicate that tocopherol, incorporated into cell membranes, suppresses the lipid peroxidation of membranes induced by CumOOH. Similar effects observed with the preincubation with probucol suggest the probucol could also be transported and incorporated into EC membranes during preincubation. This hypothesis was confirmed by our determination of the probucol content in the cells treated with probucol. With 5 mM CumOOH there was a small degree of protection against LDH release afforded by antioxidants, whereas the formation of TBARS was reduced to a similar amount with 2 mM CumOOH. The difference in the extent of the protection between the LDH release and TBARS formation...
afforded by the antioxidants may indicate the possibility of the existence of mechanisms of CumOOH-dependent lethal injury other than membrane lipid peroxidation. As described above, CumOOH easily penetrates cellular membranes and is cleaved to produce oxygen centered radicals (25, 26). These radicals generated intracellularly may directly react with cellular structures, including proteins, nucleotides, or other biologically important materials (27). In the case of damage to cell constituents other than membrane lipids, antioxidants cannot provide protection for cells.

We also found that pretreatment with either probucol or tocopherol protected EC against OX-LDL toxicity. Incubation with OX-LDL increased the TBARS content in the cells. When EC were pretreated with these antioxidants, the accumulation of TBARS in cells induced by OX-LDL was significantly reduced. We found that the probucol content increased in cells during incubation with probucol; there was a good correlation between the content of probucol and the degree of protection against OX-LDL. More than 8 h were required under these culture conditions for sufficient levels of probucol to be achieved in the cells. These observations may also indicate that the failure to protect EC by the simultaneous addition of probucol to medium containing OX-LDL or CumOOH was due to the slow rate of incorporation of probucol into the cells. Slow incorporation of probucol by EC may, in part, have been due to insolubility of the added probucol in the medium. Our detection of probucol content in cells that were incubated with the medium containing probucol plus LPDS may imply that lipoproteins are not always necessary for the incorporation of

TABLE 2. Thiobarbituric acid-reacting substances (TBARS) in cells incubated with oxidized LDL (OX-LDL)

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Thiobarbituric Acid-Reactive Substances (TBARS)</th>
<th>nMol MDA/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Cells</td>
<td>Tocopherol-Treated Cells</td>
</tr>
<tr>
<td>None</td>
<td>0.44 ± 0.11</td>
<td>0.43 ± 0.08</td>
</tr>
<tr>
<td>OX-LDL, 300 µg cholesterol/ml</td>
<td>3.52 ± 0.20</td>
<td>1.08 ± 0.24*</td>
</tr>
<tr>
<td>OX-LDL, 600 µg cholesterol/ml</td>
<td>4.76 ± 0.25</td>
<td>1.56 ± 0.34*</td>
</tr>
</tbody>
</table>

EC at confluence in T-25 flasks were incubated with MEM containing OX-LDL for 8 h at 37°C. Values represent means ± SD of triplicate flasks.

*P < 0.001 versus control cells incubated with OX-LDL (300 µg cholesterol/ml).

**P < 0.001 versus control cells incubated with OX-LDL (600 µg cholesterol/ml).
probucol into EC in vitro. It is well established that probucol is carried predominantly in the lipoprotein fractions, including LDL (28). We found that the treatment of EC with 20% of serum from patients being administered probucol resulted in the detection of probucol in the cells. These results may perhaps indicate that probucol incorporates into the EC layer through lipoproteins during the clinical administration. It has been reported that probucol blocks both the cell-mediated and copper ion-mediated oxidative modification of LDL in vitro (9). Furthermore, our observations point to the possibility of probucol being transported and incorporated in cellular membranes where it acts as a radical-trapping antioxidant, protecting the EC against injury induced by various forms of oxidative stress.

In addition, our findings explain at least part of the mechanism of EC injury inflicted by OX-LDL. It has been demonstrated that the cytotoxicity of OX-LDL does not require LDL receptor (29) on target cells, and that the toxin associated with OX-LDL resides in the lipid phase (29). The susceptibility to OX-LDL is dependent on the phase of the cell cycle (30) and on the level of intracellular glutathione (31). However, the detailed mechanism of cell injury induced by OX-LDL and the identity of the toxic substance(s) are unknown. Our finding of the protective effects of antioxidant supplementation on the toxicity of OX-LDL to EC and on the accumulation of TBARS in the cells incubated with OX-LDL indicates that the free radical reaction in EC membranes, especially lipid peroxidation, involves cell injury inflicted by OX-LDL. This hypothesis is not inconsistent with our recent report that intracellular glutathione represents a defense mechanism against OX-LDL toxicity (31). It should be noted that EC treated with OX-LDL had a greater content of TBARS than cells treated with CumOOH, and that the extent of the protective effect of antioxidant against OX-LDL was higher than the effect against CumOOH. These observations may indicate that the mechanism of EC injury caused by OX-LDL is strictly dependent on membrane lipid peroxidation as compared with the injury caused by CumOOH.

However, it is not clear how OX-LDL can induce lipid peroxidation in cellular membranes. Our observation of only a limited effect of the supplementation of liposoluble radical scavengers such as tocopherol or probucol to an OX-LDL-containing medium on preventing OX-LDL toxicity is consistent with the previous finding of Morel, Hessler, and Chisolm (32). Those investigators found that the combined addition of BHT, which can inhibit LDL oxidation, and OX-LDL to the culture medium did not alleviate the cytotoxicity of OX-LDL. This may imply that lipid (peroxyl or alkoxyl) radicals which ought to be trapped by these radical scavengers before they attack the membranes from outside do not participate in the induction of lipid peroxidation in cellular membranes as suggested by a previous report (30). Furthermore, our observation that Trolox C (a water-soluble tocopherol analog) could not effectively prevent OX-LDL toxicity excludes the possibility of the involvement of radicals in the aqueous phase in the initiation of membrane lipid peroxidation. However, if these radicals (lipid radicals or radicals in the aqueous phase) were generated from OX-LDL in an unknown manner in contact with the surface or on the surface of cellular membrane of the target cells, the radicals could attack the membrane, inducing lipid peroxidation in membrane lipids. Further work is required to reveal the precise mechanisms that lead to lipid peroxidation by OX-LDL in cellular membranes.

In conclusion, our results suggest that the antiatherogenic effect of probucol may be due, in part, to the protection against endothelial injury induced by oxidative stress by OX-LDL and hydroperoxide.

We wish to thank Dr. Richard L. Jackson for his suggestions and comments. In addition we thank Dr. Takao Kaneko of Tokyo Metropolitan Institute of Gerontology for providing Trolox C, and M. Yoshimura, S. Aoyama, and M. Doi for their technical assistance.

Manuscript received 28 September 1989, in revised form 31 January 1990, in re-revised form 31 July 1990, and in re-re-revised form 5 November 1990.

REFERENCES
8. Quinn, M. T., S. Parthasarathy, L. Fong, and D. Steinberg.

Kazuya et al. Probucol prevents oxidative injury to endothelial cells 203