Evidence for an Additional Intracellular Site of Action of Probucol in the Prevention of Oxidative Modification of Low Density Lipoprotein

Use of a New Water-soluble Probucol Derivative

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Abstract

Oxidative modification of low density lipoprotein (LDL) renders it more atherogenic. Probucol, a highly nonpolar antioxidant, is transported in lipoproteins, including LDL, and inhibits oxidative modification of LDL in vitro. The ability of probucol to inhibit atherogenesis in the LDL receptor-deficient rabbit has been attributed to its antioxidant effect. We report synthesis of a new water-soluble analogue of probucol that is very effective in preventing cell-induced LDL oxidation. The polar probucol derivative, diglutaryl probucol, is efficiently taken up by endothelial cells and macrophages in culture and is hydrolyzed to release the active antioxidant, probucol. The treated cells, after thorough washing, show a marked decrease in their capacity to oxidize LDL during a subsequent incubation. At high concentrations of the derivative, the cells also released free probucol into the medium. Thus, the effectiveness of probucol in vivo may be related both to its presence in LDL, acting as a nonspecific antioxidant, and to an additional ability to inhibit cell-mediated oxidation of LDL by virtue of its uptake into cells. (*J. Clin. Invest.* 1992. 89:1618–1621.) Key words: atherosclerosis • macrophages • foam cells • oxidized LDL • lipid peroxidation

Introduction

Recent evidence suggests that the atherogenic effects of LDL may be in part mediated through its oxidative modification (1). Probucol, originally introduced for clinical use as a hypocholesterolemic drug, has been shown to possess potent antioxidant properties and to block oxidative modification of LDL (2). It also reduces the uptake of injected native LDL by arterial macrophages in vivo (3), presumably by inhibiting oxidative modification of LDL, and slows the development of arterial lesions in LDL receptor-deficient rabbits (3, 4). Probucol is predominantly transported in lipoproteins (5) and thus may protect LDL as a "resident" antioxidant within the LDL particle. Lipoproteins also serve the function of delivering lipophilic antioxidants and vitamins to cells. The possibility that probucol may, in addition, affect cell function has also been considered. For example, Yamamoto and co-workers (6) reported that certain monocyte/macrophage cell lines preincubated with probucol showed a decrease in their ability to take up and degrade acetylated LDL. However, others have not been able to confirm that finding (3, 7) using peritoneal macrophages. Ku et al. (8) have reported that resident peritoneal macrophages from probucol-treated animals, in contrast to probucol-added macrophages, show a decrease in interleukin 1 release when stimulated by lipopolysaccharide. Recently it was reported that probucol incorporated into endothelial cells protect the cells against oxidative stress (9). In vitro studies of the effects of probucol on cell metabolism are made difficult by the fact that its cellular uptake is poor and unpredictable. For example, Kazuya et al. (9) observed an incorporation of less than 1 μg of probucol/10^6 cells when endothelial cells were incubated for 24 h with 50 μM probucol. Probucol is a highly nonpolar molecule and its solubility is very limited. For the present studies we have synthesized a new analogue of probucol that is highly polar, readily soluble in water at neutral pH, and releases active probucol upon hydrolysis. This new derivative diglutaryl probucol, is readily synthesized and should be useful for loading cells with the drug in vitro. We show that cultured endothelial cells and mouse peritoneal macrophages previously incubated with diglutaryl probucol show a marked inhibition of their ability to oxidatively modify LDL in a subsequent incubation.

Methods

The sources of cell culture supplies and media have been described earlier (10, 11). LDL was isolated and oxidatively modified as previously described, by incubation with cultured rabbit aortic endothelial cells, with mouse peritoneal macrophages or with 5 μM copper in Ham's F-10 medium (2, 10, 11). Biological modification of LDL was measured in terms of the increase in the rate of its degradation by mouse peritoneal macrophages (2, 10, 11). The water-soluble analogue of probucol was synthesized as follows: Probucol was treated with a 40-fold molar excess of glutaric anhydride in the presence of catalytic amounts of 4-dimethyl-aminopyridine at 130°C for 24 h. The formation of probucol diglutarate was monitored by thin layer chromatography (TLC) (*n*-hexane:diethyl ether:acetic acid, 70:30:1 vol/vol/vol). The product was purified by silicic acid column chromatography using increasing amounts of ether in hexane. The purified product gave a single spot on TLC (Rf 0.18) distinct from the parent compound (Rf 0.68). Upon alkaline hydrolysis the compound yielded free probucol. The water-soluble analogue, in its acid form, is soluble in organic solvents; upon removal of the solvent it can be dissolved in sodium bicarbonate solution which can be put through a 0.45-μM filter to ensure sterility and to remove any free probucol. The sodium salt in aqueous solution undergoes slow hydrolysis yielding free probucol.

[^{14}C]Probucol (gift from Marion Merrel Dow Pharmaceuticals, Cincinnati, OH) was also converted to the diglutarate derivative at a specific activity of 1 μCi/μmol. The product gave a single radioactive spot on TLC.

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Results and Discussion

The effect of preincubation with 500 nmol/ml of probucol (added in ethyl alcohol) on the ability of macrophages to oxidize LDL is shown in Table I. Probucol itself has a very limited solubility and the medium in these studies was grossly milky. After several washings with Ham's F-10, the cells were then incubated with 200 μg of ^{125}I-LDL in 2 ml of Ham's F-10 for 24 h. As seen in the table, the cells that were incubated with probucol modified LDL poorly as compared to control cells not pretreated with probucol. This suggested that antioxidant enrichment of cells might afford additional protection for LDL against cell-catalyzed oxidation. However, because the probucol was obviously not in solution and might remain adsorbed to the cell surface even after washing, we could not rule out the possibility that residual probucol on the cell surface accounted for the apparent protection during the second incubation, i.e., probucol from the cell surface might transfer into the LDL during the first part of the second incubation. Our first attempt to get around this difficulty was to use acetyl LDL, which is readily taken up by macrophages, as a vehicle for introducing probucol into the cells. However, the results varied from preparation to preparation and it was difficult to demonstrate measurable quantities of probucol in the cells. At this point, we turned to the synthesis of a water-soluble analogue, diglutaryl probucol, synthesized as described in Methods.

In preliminary experiments, the effects of diglutaryl probucol were studied using a protocol like that used previously for studying the effects of probucol itself (2), i.e., LDL was incubated with endothelial cells for 24 h either in the presence or absence of the inhibitor. Oxidative modification of LDL was strongly inhibited by even very low concentrations of diglutaryl probucol (more than 50% at 2.5 μM) but it was found that the diglutaryl probucol had been almost completely hydrolyzed in the course of the 24-h incubation, i.e., the medium at the end of the incubation contained exclusively probucol itself. Thus it was not possible to determine to what extent the observed inhibition reflected the uptake of diglutaryl probucol into the cells, on the one hand, and the effects of free probucol generated by hydrolysis of the diglutaryl derivative during the incubation, on the other. Similar experiments were done using copper-induced oxidation and with similar results (data not shown).

In the next series of studies, cells were incubated with diglutaryl probucol for only a short period of time (3 h) to limit the extent of spontaneous hydrolysis. Then the cells were thoroughly washed to remove any extracellular inhibitor. These pretreated cells were then incubated with labeled LDL for an additional 24 h. As shown in Table II, the pretreated cells were strongly inhibited with respect to their ability to induce LDL oxidation, measured either in terms of thiobarbituric acid reactive material or in terms of the biological modification (i.e., the increase in the rate of subsequent LDL degradation in a 5-h incubation with macrophages). Concentrations as low as 10 μM diglutaryl probucol inhibited the modification completely.

To rule out the possibility that glutaric acid released from the derivative might in some fashion be responsible, cells were incubated with equivalent molar concentrations of sodium glutarate. This did not inhibit the modification of the LDL. As a control on the efficiency of the washing procedures used, endothelial cells were incubated with probucol itself (100 nmol) for 3 h, washed, and then used in a subsequent incubation with labeled LDL as described above. These cells were able to oxidatively modify LDL normally, i.e., there had not been enough uptake of probucol itself during a 3-h incubation to affect the ability of the cells to oxidize LDL nor was there

Table I. Pretreatment of Macrophages with Probucol Inhibits the Cell's Ability Oxidize LDL

	TBARS	Macrophage degradation
	nmol/mg protein	*μg/5 h per mg cell protein*
Native LDL	2.6	1.1
LDL incubated with control macrophages	48.4	6.7
LDL incubated with probucol-pretreated macrophages	11.2	2.1

Overnight cultured mouse peritoneal macrophages (3×10^6 cells per dish in a 6 well plate) were treated with 500 nmol probucol in 1 ml of DME containing 5 mg/ml lipoprotein deficient serum. Probucol was added in 10 μl ethanol. After 48 h, the cells were washed 3 times with 3 ml of Ham's F-10 and then subjected to incubation with ^{125}I-LDL (100 μg/ml) in 2 ml of Ham's F-10 for 20 h. TBARS and subsequent macrophage degradation were determined. The results are averages from a triplicate determination from a representative set.

Table II. Effect of Preincubation of Endothelial Cells (EC) and Mouse Peritoneal Macrophages with Water-Soluble Probucol Derivative on the Subsequent Modification of LDL

	TBARS	Macrophage degradation
	nmol/mg protein	*μg/5 h per mg cell protein*
Set A		
Native LDL	5.5	1.6
LDL incubated with:		
Control EC	52.5	7.2
EC pretreated with:		
25 μM diglutaryl probucol	12.1	1.5
50 μM diglutaryl probucol	6.5	1.3
100 μM probucol	42.5	6.4
Set B		
Native LDL	3.2	1.5
LDL incubated with:		
Control macrophages	21.8	5.5
Macrophages pretreated with:		
10 μM diglutaryl probucol	5.3	2.2
20 μM diglutaryl probucol	4.1	1.6
30 μM diglutaryl probucol	3.3	1.4

Diglutaryl probucol (sodium salt) or probucol (in ethanol) were added to washed endothelial cells or overnight cultured macrophages in 1 ml of Ham's F-10 medium and the cells were incubated for 3 h at 37°C. The cells were washed three times with 3 ml of F-10 containing 10% fetal calf serum and were then incubated with labeled LDL as described in Table II in Ham's F-10 medium without serum. The values given are from a typical experiment from three or more separate experiments.

enough probucol adsorbed to the cells to protect the LDL by transfer into it.

The uptake of probucol glutarate by endothelial cells and macrophages was studied using ^{14}C-labeled water soluble derivative. More than 96% of the labeled derivative readily went into solution as the sodium salt and when incubated with macrophages was effectively taken up by the cells. About 25–30% of the added radioactivity (2.5–6.5 nmol of probucol/~ 40 μg of cell protein) was associated with the cells after 2 h (Table III). At 60 min about half of the cell associated radioactivity was in the form of the precursor, probucol diglutarate.

It was still necessary to consider the possibility that free probucol generated during the 3-h incubation or generated by hydrolysis in the cell might find its way into the LDL particle and act as an antioxidant in the medium. We incubated endothelial cells with 25 nmol of ^{14}C-labeled diglutaryl probucol for 3 h and after washing fresh medium and LDL were added. The LDL recovered from the medium showed absence of oxidation but was readily modified upon a subsequent incubation in the presence of 5 μM copper. However, when higher concentrations 50–200 nmol of diglutaryl probucol were incubated with endothelial cells, there was considerable release of free probucol into the medium (in a 24-h incubation) even after several washings with medium containing lipoprotein-deficient medium. Nevertheless, after two subsequent incubations with LDL at 100 μg/ml for 24 h each, 30–45% of the incorporated radioactivity was still associated with the cells. It should be pointed out that in these experiments, more than 15 nmol of probucol was incorporated into the cells of which about 7 nmol were released into the medium during a 24-h incubation with LDL. The LDL recovered from such incubations was resistant to modification upon a subsequent incubation with 5 μM copper. Thus, cells enriched in probucol, also released the antioxidant into the medium which may offer additional protection against oxidation. The rate of release of probucol from cells was not followed in these studies.

Although the presence of probucol in LDL clearly protects it to some extent against oxidative modification, by acting as a relatively nonspecific antioxidant within the LDL particle (2), the present results suggest an additional mode of action that may be relevant to the in vivo effects of probucol. While the rate of entrance of probucol into cells in culture is slow, the cells of animals treated chronically with the drug may take up enough of it so that their metabolism is altered, most specifically, their ability to oxidatively modify LDL. Probucol has been reported to accumulate in several tissues at concentrations even higher than in plasma (12). Other studies from this laboratory (13–16) have implicated lipoxygenases in the oxidative modification of LDL. We have proposed that the lipoxygenases act initially on cell lipids to generate hydroperoxides of fatty acids which are then transferred to the LDL. Probucol within the cell might prevent the generation of such lipoperoxides either by acting directly on the lipoxygenase systems or by limiting propagation reactions within the cell membrane. Cells may also release stored probucol into the extracellular medium, thus limiting lipid peroxidation. These findings suggest still another strategy for inhibition of oxidative modification of LDL, i.e., the introduction of compounds into cells to inhibit their ability to induce LDL oxidation. The combination of an antioxidant within the LDL molecule and the presence of an inhibitor within the cells might be additive. Thus, the antiatherogenic effects of probucol (3, 4) may very well depend upon such a two-pronged mode of action.

In summary, this derivative offers a convenient, water-soluble, filter-sterilizable means of delivering a pro-drug that is efficiently taken up by cells and releases free probucol upon hydrolysis.

Table III. Uptake of ^{14}C-labeled Probucol Diglutarate by Macrophages

Cell type	Nmol ^{14}C-diglutaryl probucol added	Nmol of cell Associated ^{14}C-Radioactivity
Macrophages	5	2.5
	10	3.7
	15	4.2
	20	5.2
	25	6.5
Endothelial cells	5	1.9±0.14

Sodium salt of ^{14}C-diglutaryl probucol was added to washed cells at the specified concentrations and incubated in 1 ml of DME medium at 37°C for 3 h. The cells were then washed three times with DME. (The medium after the last washing did not contain any radioactivity.) The cells were dissolved in 1 ml of 0.01% Triton X-100 before the determination of radioactivity. Values for macrophages represent averages of duplicate determinations from one of two separate trials. Values for endothelial cells are from four individual cell incubations.

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