Original Article

Effect of Probucol on Antioxidant Properties of HDL in Patients with Heterozygous Familial Hypercholesterolemia

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Aim: High density lipoprotein (HDL) has multi-antiatherogenic effects such as antioxidation and anti-inflammation, in addition to being a key mediator of reverse cholesterol transport. Probucol, known as a lipid lowering drug, is also a potent antioxidant, but it decreases serum HDL cholesterol (HDL-C) levels. To elucidate the effect of probucol on antioxidant properties of HDL, we investigated the function of HDL derived from patients with heterozygous familial hypercholesterolemia (FH) who have been treated with probucol.

Methods and Results: Probucol-treated FH patients ($n=21$) showed a 47% reduction of serum HDL-C levels compared to probucol-untreated FH patients ($n=15$). High performance liquid chromatography (HPLC) analysis revealed that probucol diminished HDL particle size compared to the non-treated group. Antioxidant capacity of HDL was evaluated by its effect to protect reference LDL from oxidation induced in the presence of an oxidizing agent, AAPH. The HDL derived from the probucol-treated group demonstrated a significantly prolonged time to start oxidation by 112%, decreased the maximum oxidation rate by 14%, and lowered the maximum concentration of conjugated dienes formation by 15%. Furthermore, HDL-associated paraoxonase 1 (PON1) activity, but not platelet-activating factor acetyl-hydrolase (PAF-AH) correlated with these measurements of HDL anti-oxidative activity. Treatment with probucol *in vitro* and inhibition of PON1 activity demonstrated that probucol in HDL particles and increase of PON1 activity might largely contribute to the increase of HDL anti-oxidative activity.

Conclusion: Probucol reduced HDL-C levels and HDL particle size in patients with heterozygous FH, while it concomitantly enhanced HDL anti-oxidative properties, possibly through increasing PON1 activity.

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Key words; HDL, Probucol, Familial hypercholesterolemia, Antioxidant property, PON1

Introduction

HDL particles possess multiple anti-atherogenic activities, such as 1) their capacity to mediate cellular

cholesterol efflux by acting as primary acceptors, thereby facilitating reverse cholesterol transport (RCT) from the arterial wall and peripheral tissues to the liver, 2) protection of LDL against oxidative stress, 3) anti-inflammatory actions on arterial wall cells, and 4) anti-apoptotic, 5) vasodilatory, 6) antithrombotic, and 7) anti-infectious activities.

Probucol is a bisphenolic compound with unique antiatherogenic attributes including LDL-C-lowering, antioxidant, and anti-inflammatory properties.

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Administration of probucol also leads to regression of skin and tendon xanthomas in patients with familial hypercholesterolemia (FH), despite reduced serum HDL-C levels¹⁾. Probucol has often been used in Japan, and recently was demonstrated to reduce coronary artery disease (CAD) risks significantly in patients with heterozygous FH who have a very high incidence of CAD. Accordingly, the unique antiatherogenic feature of this old and often misunderstood drug has drawn physicians' attention²⁾. Some elements of probucol's antiatherogenic effects have been explained by its antioxidative effects against LDL³⁾. In addition, we reported previously that the extent of regression of Achilles tendon thickness was closely correlated with the reduction in serum HDL-C in patients with FH during probucol treatment^{4, 5)}. We also reported that small HDL particles from probucol-treated patients are more potent than untreated HDL in removing cholesterol from peripheral tissues⁶⁾, that RCT was enhanced by stabilizing the expression of hepatic SR-BI (scavenger receptor class B, type 1)⁷⁾, and that long-term probucol treatment prevented secondary cardiovascular event in patients with heterozygous FH⁸⁾; however, previous studies did not investigate whether probucol treatment changes the antioxidative properties of HDL.

Major HDL enzymes related to antioxidative activity against LDL oxidation are paraoxonase 1 (PON1)⁹⁾ and platelet-activating factor acetylhydrolase (PAF-AH)¹⁰⁾.

PON1 is synthesized in the liver and is almost exclusively located on plasma HDL. The enzyme inhibits atherogenesis by preventing the oxidation of both HDL and LDL^{11, 12)}. In contrast, the HDL isolated from PON1 KO mice failed to prevent LDL oxidation¹³⁾. Some studies have indicated that low PON1 activity is a strong risk factor for CAD¹⁴⁻¹⁶⁾. Probucol up-regulates PON1 mRNA expression in hepatocytes of hypercholesterolemic rabbits¹⁷⁾. Plasma PAF-AH might play an anti-inflammatory role in human disease by preventing the accumulation of PAF and PAF-like oxidized phospholipids; however, the role of this enzyme in atherosclerosis is still controversial¹⁸⁾.

In the current study, we investigated the effects of probucol on antioxidative properties of HDL in patients with heterozygous FH and assessed the association of the antioxidative activity of HDL with HDL-associated enzymes.

Methods

Subjects

Thirty-six subjects with heterozygous FH (pro-

bucol treated: $n=21$, probucol non-treated: $n=15$) were enrolled in this study. The duration of probucol treatment was about 19.8 ± 10.5 years. Heterozygous FH was defined as having at least two major features, or at least one major and one minor feature. The major features of FH are total cholesterol (TC) of 260 mg/dL and above; tendon xanthoma or tuberous xanthoma; reduced or abnormal receptor activity noted by LDL receptor analysis, and the minor features are palpebral xanthoma; juvenile (<50 years) corneal arcus; juvenile (<50 years) ischemic heart disease⁸⁾. Eight healthy normolipidemic volunteers without a history of hypercholesterolemia or CAD were enrolled as control subjects. All subjects gave their informed consent before entering the study according to Osaka University Hospital Ethics Committee, and none of our blood donors was taking antioxidant vitamin supplementation. Venous blood was drawn after overnight fasting. Serum was separated by low-speed centrifugation (3000 rpm, 30 minutes, at 4°C) and aliquots were frozen at -80°C under nitrogen until measurement.

Isolation of Serum Lipoproteins

Serum lipoproteins were fractionated by sequential ultracentrifugation. HDL was isolated in the density range 1.063-1.210 g/mL and LDL was isolated in the density range 1.019-1.063 g/mL. HDL and LDL were each mixed with sucrose (final concentration, 10%) as a cryoprotectant for lipoproteins and frozen at -80°C under nitrogen. Before use, KBr and EDTA were removed from LDL and HDL fractions by extensive dialysis with Dulbecco's phosphate buffer solution (PBS) for 48 h at 4°C. Polyethylene glycol (PEG) precipitation method¹⁹⁾ was used for isolation of HDL-associated antioxidative enzyme activity for protection of HDL composition against exposure to extreme g-forces in the process of ultracentrifugation²⁰⁾.

Characterization of Serum Lipoproteins

Concentrations of TC, triglyceride (TG), LDL-C and HDL-C were measured by an enzymatic method. Concentrations of apolipoproteins (apo) A-I, A-II, B, C-II, C-III, and E were measured by an immunoturbidimetric method. The lipoprotein profiles of the patients and controls were analyzed using an online dual enzymatic method for simultaneous quantification by HPLC at Skylight Biotech Inc. (Akita, Japan), according to the procedure described by Usui *et al.*²¹⁾.

Antioxidative Activity of HDL

Antioxidative activity of HDL toward reference LDL isolated from control subjects was assessed.

Fig. 1. Typical absorption time course at 234 nm (CD formation) produced during oxidation of LDL by AAPH.

The kinetics of CD accumulation revealed two characteristic phases, the lag and propagation phases. For each kinetic curve, the duration of the lag phase, average oxidation rates within the propagation phase and amount of CD formed at the end of the propagation phase (maximal amount of CD) were evaluated.

Before oxidation, EDTA and KBr were removed from LDL and HDL by dialysis for 48 h at 4°C. LDL (60 µg protein/mL) was incubated at 37°C in PBS (pH 7.4) in the presence of 1 mM AAPH (2, 2-azobis-(2-amidinopropane) hydrochloride); HDL (100 µg protein/mL) was added to LDL immediately. Accumulation of CD in the process of LDL oxidation was measured as the increment in absorbance at 234 nm²²⁾. The kinetics of CD accumulation revealed two characteristic phases, the lag and propagation phases. For each kinetic curve, the duration of the lag phase, average oxidation rates within the propagation phase and the amount of CD formed at the end of the propagation phase (maximal amount of CD) were evaluated (Fig. 1).

Measurement of HDL-Associated Antioxidative Enzyme Activity

Analysis of PON1 Activity

PON1 activity in HDL was assessed in arylesterase and homocysteine thiolactonase (HTLase) activities. Arylesterase activities are significantly affected by polymorphisms of the PON1 gene; however, HTLase activity does not usually differ between the polymorphisms²³⁾. Arylesterase activity was estimated using a Zeptometrix Assay kit (Zeptometrix Corp, New York)

based on the cleavage of phenyl acetate, resulting in phenol formation. The formation rate of phenol was measured by monitoring the increase in absorbance at 270 nm at 25°C. HTLase activity was evaluated by an HTLase assay kit (Alfresa Auto HTLase; Alfresa Pharma Corp., Japan). This method utilizes gamma-thiobutyrolactone as a substrate and Ellman's procedure to monitor the accumulation of free groups via coupling with 5, 5-dithiobis (2-nitrobenzoic acid)²³⁾.

Analysis of PAF-AH Activity

PAF-AH activity was measured by the Azwell Auto PAF-AH kit (Azwell Inc., Japan). PAF-AH hydrolyzes the sn-2 position of the substrate [1-myristoyl-2- (4-nitrophenyl succinyl) phosphatidylcholine], producing 4-nitrophenylsuccinate, which is immediately degraded to 4-nitrophenol and subsequently measured spectrophotometrically²⁴⁾.

Effects of Probucol *in vitro* on Antioxidative Activities of HDL

Probucol (40 mM) was diluted in DMSO. This solution was added to HDL from probucol-untreated patients, and incubated 24 h at 4°C. Final concentration of probucol was 50 µM. Probucol-treated HDL was dialyzed for 48 h at 4°C, and antioxidative activity

Table 1. Patients Baseline Clinical Characteristics, Lipid, and Lipoprotein Profiles

	Probucol (+)	Probucol (–)	<i>p</i> value
N	21	15	
Age, y	66 ± 14	48 ± 13	<0.001
Men (%)	48	33	0.9
Current smoking	1 (5%)	2 (13%)	0.22
Statins (%)	100%	100%	0.21
Resins	7 (33%)	2 (13%)	0.37
Ezetimibe	5 (31%)	3 (20%)	0.83
Fibrates	1 (5%)	0 (0%)	0.46
BMI, kg/m ²	22 ± 3	22 ± 3	0.32
SBP, mmHg	133 ± 15	128 ± 15	0.17
DBP, mmHg	77 ± 10	80 ± 9	0.19
Total cholesterol, mg/dL	186 ± 38	211 ± 30	<0.05
Triglycerides, mg/dL	66 ± 29	74 ± 31	0.22
HDL-C, mg/dL	34 ± 10	64 ± 12	<0.0001
LDL-C, mg/dL	137 ± 32	129 ± 30	0.24
Apolipoprotein A-I, mg/dL	92 ± 23	148 ± 14	<0.0001
Apolipoprotein A-II, mg/dL	25 ± 5	31 ± 5	<0.001
Apolipoprotein B, mg/dL	111 ± 20	100 ± 19	0.06
Apolipoprotein C-II, mg/dL	2 ± 1	3 ± 1	0.1
Apolipoprotein C-III, mg/dL	7 ± 2	9 ± 2	<0.05
Apolipoprotein E, mg/dL	4 ± 1	4 ± 1	0.38

SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Data are shown as the mean ± SD and statistical significance was calculated by paired *t*-test.

of HDL was measured. As a control, HDL was incubated with DMSO at a final concentration of 0.125%. The results are shown as the mean of duplicated data.

Inhibition of PON1 Activity

HDL from patients was dialyzed for 48 h at 4°C. A specific inhibitor of PON1^{25, 26)}, 2-hydroxyquinoline (Sigma-Aldrich Corp., St. Louis, MO) was diluted in methanol (10 mM). This stock solution was diluted in PBS. HDL was incubated with 2-hydroxyquinoline (5 μM), and antioxidative activity of HDL was measured. As a control, HDL was incubated with methanol at a final concentration of 0.05%. The results are shown as the mean of duplicated data.

Statistical Analysis

Data are shown as the means ± SD. Statistically significant differences were determined by Kruskal-Wallis tests followed by Dunn's multiple comparison post-test, one-way ANOVA tests followed by Tukey's multiple comparison post-test, or a two-tailed, unpaired Student's *t*-test. Distribution of categorical variables was analyzed by Fisher's exact test. Pearson's correlation coefficients were calculated to evaluate

relationships between variables. A value of *p* < 0.05 was considered significant.

Results

Clinical Characteristics of the Subjects Investigated

Twenty-one probucol-treated patients and 15 untreated patients with heterozygous FH participated in this study. There was a significant difference in age between the two groups; however, there were no significant differences in the body mass index (BMI), sex distribution, smoking habit, other lipid-lowering drugs, and blood pressure. Probucol-treated FH patients showed a 47% reduction of serum HDL-C compared to probucol-untreated FH patients. Serum total cholesterol, apo A-I, apo A-II, and apo C-III were significantly lower in the probucol-treated group than in the probucol-untreated group. In contrast, LDL-C levels were not significantly different between the two groups (Table 1).

HPLC Analysis

The chromatographic patterns of each lipoprotein obtained from the mean value of probucol-treated

Fig. 2. Chromatographic patterns of each lipoprotein obtained with the mean value in probucol-treated patient ($n=21$, solid line) and probucol-untreated patient ($n=15$, dotted line) are shown. A: mV of total cholesterol (TC), B: mV of triglyceride (TG), C: mV of free cholesterol (FC), D: mV of phospholipids (PL). Arrows show the peak HDL fraction.

patients (solid line) and probucol-untreated patients (dotted line) are shown in **Fig. 2**. Arrows show the peak HDL fraction. The HDL peaks on solid lines shift to the right as compared with dotted lines. This result indicated that HDL particles became smaller in the probucol-treated group than in the probucol-untreated group.

Antioxidative Function of HDL during LDL Oxidation

Influence of HDL on LDL oxidation by AAPH is shown in **Fig. 3**. When the HDL isolated from probucol-treated patients or probucol-untreated patients was added to the reference LDL at a physiological HDL to LDL ratio of about 2-4 mol/mol, LDL oxidation was significantly delayed in the probucol-treated group. HDL from the probucol-treated group significantly prolonged the time to start oxidation (lag phase duration) by 112% compared with HDL from the probucol-untreated group, while there was no significant difference between HDL from the probucol-untreated group and control subjects (**Fig. 3A**). Furthermore, HDL from the probucol-treated group significantly decreased the maximum oxidation rate by

14% in the propagation phase (**Fig. 3B**) and decreased maximal amounts of CD formation by 15% (**Fig. 3C**) compared with HDL from the probucol-untreated group. There was no significant difference between HDL from the probucol-untreated group and control subjects regarding the maximum oxidation rate and maximal concentrations of CD. Thus, HDL derived from probucol-treated patients was more resistant to LDL oxidation induced by AAPH.

Antioxidative Enzyme Activity Associated with HDL

Fig. 4 illustrates HDL-associated protein activity corrected by apoA-I concentration. PON1 activities in terms of arylesterase and HTLase were significantly increased in the probucol-treated group compared with the probucol-untreated group, while HDL-associated PAF-AH activity was not significantly different between the groups.

The relationship between the antioxidative activity of HDL and HDL-associated protein activity is presented in **Fig. 5A-F**. The lag phase duration was positively correlated with PON1 activity measured by arylesterase activity and HTLase activity (**Fig. 5A** and **5B**). Furthermore, the LDL maximal oxidation rate

Fig. 3. Influence of HDL derived from patients on LDL oxidation by AAPH.

(A) Lag phase duration, (B) LDL oxidation rate in propagation phase, (C) Maximum concentration of conjugated dienes are shown for probucol-treated ($n=21$, white bar) and untreated ($n=15$, black bar) patients, and the control ($n=8$, gray bar). HDL from probucol-treated group showed significantly prolonged lag phase, decreased maximum oxidation rate, and decreased maximal amounts of CD formation. ** $p < 0.01$. *** $p < 0.001$

(Fig. 5C and 5D) and maximal concentration of CD (Fig. 5E and 5F) were negatively correlated with PON1 activity; however, no significant correlation was found between the antioxidative activity of HDL and PAF-AH activity (Table 2).

Effects of Probucol *in vitro* on Anti-oxidative Activities of HDL

To elucidate the contribution of probucol contained in HDL to the anti-oxidative activity of HDL, probucol was incubated *in vitro* with HDL from probucol-untreated patients (Fig. 6). Addition of probucol *in vitro* demonstrated a 27% increase of lag time, 8% decrease of maximal oxidation rate, and 11% decrease of the maximal concentration of CD.

Inhibition of PON1 Activity

To elucidate the contribution of PON1 in the enhancement of anti-oxidative activity of HDL, anti-oxidative activities of HDL were measured with or without a PON1-specific inhibitor, 2-hydroxyquinoline (Fig. 7). Lag duration time of HDL from probucol-treated patients was reduced by 40% with 2-hydroxyquinoline treatment (Fig. 7A). In HDL from probucol-untreated patients, inhibition of PON1 revealed 25% reduction of the lag time.

Discussion

Clinical Significance of Probucol

Probucol is known to have significant effects of rapid and marked regression of xanthoma and Achilles tendon thickening in patients with FH despite a

Fig. 4. HDL-associated PON1 and PAF-AH activities corrected by apoA-I concentration.

Arylesterase activity (A), HTLase activity (B), and PAF-AH activity (C) are shown for probucol-treated patients ($n=21$, white bar), untreated patients ($n=15$, black bar), and the control ($n=8$, gray bar). PON1 activities in terms of arylesterase and HTLase were significantly increased in the probucol-treated group while PAF-AH activity was not significantly different among the groups. *** $p < 0.001$.

reduction of serum HDL-C levels. One of probucol's mechanisms for xanthoma regression was shown to involve the suppression of oxidative modification of LDL²⁷. HDL was reported to have antioxidant properties²⁸⁻³⁰. Specific effects of probucol on antioxidative properties of HDL have not been reported; therefore, in the current study, we focused on the anti-oxidation effect of HDL obtained from probucol-treated FH patients.

In this study, we observed that treatment with probucol significantly decreased HDL-C by 47%, although there was no significant difference in LDL-C between the probucol-treated group and probucol-untreated group (**Table 1**). The lack of a significant difference in the LDL-C level between the two groups was probably due to the high prevalence of strong statin administration in the probucol-untreated group. Mean age of the probucol-treated group was significantly higher than that of the probucol-untreated

group; however, this difference cannot reject the conclusion that HDL from probucol-treated patients exhibited more anti-oxidative capacity, because the older group should exhibit more oxidative stress.

Probucol lowers HDL-C levels which are not associated with the risk of cardiovascular events⁸. The controversial and anti-atherogenic feature of probucol is most likely attributable to changes in HDL properties and their molecular mechanisms. Probucol lowers serum HDL-C, but enhances RCT by activation of cholesteryl ester transfer protein (CETP)⁶. We previously reported that the HDL particles of probucol-treated FH patients are efficient in promoting cholesterol efflux, thereby enhancing RCT⁴. HDL particles obtained from probucol-treated patients were significantly smaller than those of control HDL because of the enhancement of RCT, as reported previously³¹. In fact, our current data were consistent with those reported previously (**Fig. 2**).

Fig. 5. Correlations between antioxidative activities of HDL and PON1 activities.

Correlations between PON1 activity measured by arylesterase activity (A, C, E) or HTLase activity (B, D, F) and lag phase duration (A, B) or LDL oxidation rate in the propagation phase (C, D) or maximal concentration of CD (E, F). PON1 activities significantly correlated with antioxidative activities of HDL.

Antioxidative Effect of HDL against LDL Oxidation

In the current study, we analyzed the anti-oxidative functions of HDL from FH patients treated with probucol. Our result showed that the small HDL particles derived from the probucol-treated group significantly prolonged the lag phase duration by 112%, decreased the maximum oxidation rate by 14%, and lowered the maximum concentration of CD by 15% compared to the non-treated group (**Fig. 3**). Consequently, HDL derived from probucol-treated FH patients strongly protected LDL against oxidation.

To further explore the mechanisms of antioxi-

dative effects of probucol-treated HDL against AAPH-induced CD formation in reference LDL, we predicted the precise process to be as follows. AAPH produces free radicals at a constant rate in the presence of oxygen in aqueous solution, initiates lipid peroxidation, and increases oxidized LDL (oxLDL). In the lag phase, AAPH-induced free radicals are scavenged by antioxidants in the aqueous phase or later in the lipid phase, such as vitamin C, vitamin E, and probucol. In the propagation phase, free radicals begin to peroxidize LDL and produce CD, and concomitantly these oxidized lipids are catabolized or removed by antioxi-

Table 2. Correlation between LDL oxidation and HDL-associated protein activity in FH patients

	lag phase duration (min)		maximal oxidation rate (dA/min)		maximal conjugated dienes concentration (λ 234 nm)	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Arylesterase (kU/L/mg/dL)	0.73	<0.0001	-0.53	0.002	-0.59	0.001
HTLase (IU/L/mg/dL)	0.69	<0.0001	-0.45	0.011	-0.48	0.006
PAF-AH (IU/L/mg/dL)	0.12	0.505	-0.28	0.124	-0.3	0.095

Fig. 6. Effects of probucol *in vitro* on antioxidative activities of HDL. Antioxidative activities of HDL from probucol-untreated patients were measured after the *in vitro* treatment of probucol. (A) Lag duration time, (B) LDL oxidation rate in propagation phase, (C) Maximum concentration of conjugated dienes.

 Probucol-untreated HDL *in vitro* (black bar). Probucol-treated HDL *in vitro* (white bar).

dative enzymes or HDL. Finally, about 70-80% of the reference LDL is oxidized and maximum amounts of CD are formed. In the following paragraphs, we discuss the three mechanisms of the antioxidative effects of probucol-treated HDL.

HDL Subclasses and Antioxidation

Small HDL is known to have antioxidative properties that do not change lag time, but reduce the oxidation rate and maximum amount of CD during LDL oxidation²⁹). Apo A-I may play a central role in HDL-mediated antioxidative activity, as Met residues 112

Fig. 7. Influence of a PON1 inhibitor on antioxidative activities of HDL derived from patients. Antioxidative activities of HDL were measured with or without a PON1 inhibitor.

(A) Lag phase duration, (B) LDL oxidation rate in propagation phase, (C) Maximum concentration of conjugated dienes are shown for PON1 inhibitor-treated (white bar) and PON1 inhibitor-untreated (black bar) patients.

and 148 can reduce lipid hydroperoxides (LOOH) to redox-inactive lipid hydroxides, and lipid-free apo A-I attenuates LDL oxidation by removal of seeding LOOH molecules from LDL^{30, 32}.

Probucol increases plasma CETP activity and enhances the expression of hepatic SR-BI in humans and rabbits⁷, resulting in an increase in small dense HDL. Our results confirmed that probucol treatment produced small HDL particles in FH patients, and showed that not only the oxidation rate and maximum amount of CD but also the lag time was affected by HDL derived from FH patients treated with probucol. This suggests that other effects of probucol on HDL may exist rather than the production of small HDL particles.

Direct Effect of Probucol against LDL Oxidation

Probucol is a potent free radical scavenger. Some studies have evaluated the effects of probucol against LDL oxidation. LDL from the probucol-treated group showed a 2.7-fold prolonged lag time³³. In WHHL rabbits, probucol treatment showed a prolonged lag time against LDL oxidation; however, the oxidation rate and maximum CD production were not affected³⁴. Similar results were also confirmed in humans³⁵. We demonstrated, for the first time, the HDL-mediated favorable effects of probucol on LDL oxidation. Probucol is known to be distributed in all lipoproteins, including HDL particles, even after dialysis. Probucol in HDL particles may act to reduce free radicals induced by AAPH, leading to prolongation of the lag time.

Fig. 8. Antioxidative mechanisms of probucol via HDL.

Three main pathways are speculated; increased antioxidants on HDL by free radical scavenge effects, increased PON1 on HDL, and induction of small dense HDL.

PON1 and Antioxidation

PON1 is a protein with 354 amino acids, synthesized in the liver and physically located on HDL particles. PON1 is strongly lipophilic and requires a lipid environment to maintain its activity¹⁶. PON1 and PAF-AH have been proposed to hydrolyze short-chain oxidized phospholipids. By contrast, PON1 and PAF-AH are weakly reactive towards LOOH³⁶. Moreover, accumulating data question the ability of PON1 to hydrolyze oxidized phospholipids and suggest that PAF-AH, rather than PON-1, is the hydrolase for oxidized phospholipids in HDL³⁶⁻³⁸; however, our data suggest the importance of PON1 rather than PAF-AH against LDL oxidation. Recent proteomic studies have identified multiple proteins that are co-isolated with human HDL²⁰. Levels of specific HDL proteins, including PON1, correlated with the potent capacity of HDL3 to protect LDL from oxidation²⁰. PON1 has several different catalytic activities involving different residues and that its physiologic substrate(s) have not yet been defined³⁸. Another study indicated that the two histidine residues required for the lactonase activity of PON1 are also essential for protecting LDL against oxidative modification³⁹; therefore, the antioxidative properties of HDL may be unrelated to its capacity to inactivate LOOH, but rather may involve its major activity as a lactonase through a still unknown pathway upstream of the regulation of systemic oxidative stress³⁶.

Regulation of PON1

A variety of pharmacological agents were previously shown to affect PON1¹⁶. Atorvastatin reduced LDL-associated PAF-AH activity and induced HDL-associated PAF-AH and PON1 activities⁴⁰. Our data suggest that the HDL-mediated antioxidative effects of probucol exceed those of statins. Interestingly, it was demonstrated that probucol induced hepatic PON1 secretion in rabbits¹⁷. Noto *et al.* reported that probucol increased PON1 activity by 30% even in patients with complete CETP deficiency⁴¹. Furthermore, probucol induced hepatic SR-BI expression⁷, and SR-BI may be a major determinant of the capacity of HDL to acquire PON1³⁷. SR-BI deficiency resulted in reduced activity of the antioxidant enzyme PON1 and a significant increase in oxidative stress⁴². The present study is the first report showing a significant increase in HDL-associated PON1 activity in humans treated with probucol. The precise mechanisms of probucol in enhancing HDL-associated PON1 activity remain unclear.

Contribution of PON1 and Probucol Contained in HDL to the Anti-Oxidative Activity of HDL

Probucol contained in patient HDL can exert strong antioxidative action on LDL by itself. Addition of probucol to HDL in vitro revealed a 27% increase of lag time. This enhancement of HDL antioxidative activity was quite small compared with the increased lag time (112%) when probucol was administrated *in*

vivo (Fig. 3A). In the maximal oxidation rate and maximal concentration of CD, the *in vitro* effect of probucol (Fig. 6B, 6C) was compatible with its *in vivo* effect (Fig. 3B, 3C). These results suggest that some antioxidative substances induced by probucol may largely contribute to the prolonged lag time in HDL from probucol-treated patients. This hypothesis was confirmed by the inhibition of PON1 activity. A high throughput serum paraoxonase assay discovered that 2-hydroxyquinoline was the most specific inhibitor of PON1²⁶. First, we confirmed that treatment with 2-hydroxyquinoline inhibited 90% of PON1 activity in HDL. This inhibition rate was compatible with a previous report²⁶. The lag duration time of HDL from probucol-treated patients was reduced by 40% with 2-hydroxyquinoline treatment (Fig. 7A). In HDL from probucol-untreated patients, inhibition of PON1 revealed 25% reduction of the lag time. This means that PON1 activity may account for a large portion of the prolonged lag time, because HDL from probucol-untreated patients showed 56% reduction of lag time compared with HDL from probucol-treated patients (Fig. 3A). Probucol dissolved in HDL may account for the other portion of the prolonged lag time. As for the maximal oxidation rate and maximal concentration of CD, effects of PON1 inhibition were not detectable (Fig. 7B, 7C). Taken together, the enhanced PON1 activity by probucol treatment *in vivo* may account for a large part of the prolonged lag time, while probucol contained in HDL may account for the reduced maximal oxidation rate and maximal concentration of CD. Namely, probucol in HDL particles and increased PON1 activity may largely contribute to the increase of HDL antioxidative activity *in vivo*.

Study Limitation

The maximal oxidation rate of probucol-treated patients in Fig. 7B was lower than that in Fig. 3B. This difference could be within the variation of samples, but we could not perform further experiments because of the limited amount of serum from patients; therefore, we could not reach definitive conclusions from Fig. 6 and 7.

Because this was a cross-sectional study, the selection bias in the choice of probucol treatment cannot be excluded, and the length of treatment was different between the two groups. Prospective studies will produce more reliable data in the future.

Another problem is that probucol enhanced the antioxidative activity of HDL but reduced ApoAI levels. We cannot demonstrate which effect is predominant, because evaluation of antioxidative activities is

complicated. The increased lag time (112% increase) may overcome the decrease of ApoAI (49% decrease), but the maximum oxidation rate and maximum CD formation may not. This *in vitro* study cannot confirm that the enhancement of antioxidative activity of HDL can overcome the reduction of ApoAI levels *in vivo*.

Taken together, the three antioxidative mechanisms of probucol via HDL, induction of small dense HDL, increased antioxidants on HDL by free radical scavenge effects, and increased PON1 on HDL, are summarized in Fig. 8.

In conclusion, HDL particles from heterozygous FH patients treated with probucol exhibited more antioxidative activity than those without probucol treatment. HDL antioxidative capacity is increased by probucol at least partially through the drug itself contained in HDL particles and through induction of PON1 activity.

Abbreviations

2,2-azobis-(2-amidinopropane) hydrochloride (AAPH), Apolipoproteins (apo), Body mass index (BMI), Conjugated dienes (CD), Cholesteryl ester transfer protein (CETP), Coronary artery disease (CAD), Familial hypercholesterolemia (FH), High performance liquid chromatography (HPLC), High-density lipoprotein-cholesterol (HDL-C), Homocysteine thiolactonase (HTLase), Lipid hydroperoxides (LOOH), Low-density lipoprotein-cholesterol (LDL-C), Oxidized LDL (oxLDL), Paraoxonase1 (PON1), Platelet-activating factor acetyl-hydrolase (PAF-AH), Phosphate-buffered saline (PBS), Polyethylene glycol (PEG), Reverse cholesterol transport (RCT), Scavenger receptor class B type I (SR-BI), Total cholesterol (TC), Triglycerides (TG).

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Disclosures

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