Original Paper

Probucol Protects Endothelial Progenitor Cells Against Oxidized Low-Density Lipoprotein via Suppression of Reactive Oxygen Species Formation *In Vivo*

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Key Word

Probucol • Endothelial progenitor cells • Oxidized low-density lipoprotein • Reactive oxygen species

Abstract

Background/Aims: Oxidized low-density lipoprotein (ox-LDL) is a major component of hyperlipidemia and contributes to atherosclerosis. Endothelial progenitor cells (EPCs) play an important role in preventing atherosclerosis and notably decreased in hyperlipidemia. Ox-LDL and ox-LDL-related reactive oxygen species (ROS) have deleterious effects on EPCs. Probucol as an antioxidant and anti-inflammatory drug reduces ROS production. The present study was to determine if probucol could protect EPCs from ox-LDL *in vivo* and to investigate the potential mechanisms. **Methods:** ox-LDL was injected into male C57BL/6 mice for 3 days with or without probucol treatment with PBS as control. Bone marrow (BM) fluid, serum, circulating mononuclear cells (MNCs) and EPCs were collected for analysis. **Results:** the increased extracellular ROS in BM, serum and blood intracellular ROS production in the mice with ox-LDL treatment in association with a significant reduction of circulating MNCs and EPCs were restored with Probucol treatment. A significant increase in the serum ox-LDL and C-reactive protein and decrease in superoxide dismutase and circulating MNCs and EPCs were observed

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in hyperlipidemic patients that were effectively reversed with probucol treatment. **Conclusion:** these data suggested that probucol could protect EPCs from ox-LDL through inhibition of ROS production *in vivo*.

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Introduction

Endothelial injury or dysfunction has been considered as an initial trigger of the development of atherosclerosis [1-4]. Endothelial progenitor cells (EPCs), as an important cell population in the bone marrow and blood mononuclear cells (MNCs), play a critical role in angiogenesis, endothelial repair, vascular re-endothelialization, and prevention of neointima formation [5-7]. The number of circulating EPCs is significantly decreased in patients with atherosclerosis or coronary artery disease (CAD) [8, 9]. Oxidized low-density lipoprotein (ox-LDL), a key component in hyperlipidemic state, has been implicated in the formation of atherosclerotic plaques. Both clinical and animal studies have shown that ox-LDL level was significantly elevated in hyperlipidemia [10]. There are extensive interactions between ox-LDL and a variety of cells through multiple mechanisms. The biological effects of ox-LDL on its target cells are highly variable and complex, depending on the individual cell type [11-20]. Ox-LDL was reported to inhibit EPC proliferation, differentiation, and suppress EPCs function including inhibition of cell migration, adhesion and *in vitro* vasculogenesis as well as ischemia-induced neovascularization *in vivo* [17, 21-24].

Reactive oxygen species (ROS) plays a critical role in the development of atherosclerosis [25-27]. It was demonstrated that a significant amount of ROS was produced spontaneously from ox-LDL at clinically relevant concentrations, and was involved in the action of ox-LDL on human umbilical vein endothelial cells [28, 29], bone marrow (BM) stem cells [30], vascular smooth muscle cells [31], monocytes [32], macrophage [33] and foam cells [34]. Treatment with ox-LDL increased intracellular ROS generation in cultured endothelial cells [35]. Our previous study showed that intravenous injection of ox-LDL could induce ROS formation in murine BM and blood [36]. It is well known that antioxidant enzyme superoxide dismutase (SOD) could attenuate ROS generation *in vivo* and *in vitro* [36], while SOD deficiency leads to increased ROS production and endothelial dysfunction with nitroglycerin-induced tolerance [37]. Plasma C-reactive protein (CRP) is known as a pro-inflammatory marker [38], and is associated with ROS generation [39] and SOD activities [40]. Therefore, SOD and CRP were used as an indirect evidence for ROS production as well.

Probucol, as a diphenolic compound with anti-oxidant and anti-inflammatory properties, could decrease atherosclerosis and restenosis in coronary arteries [41]. It has been reported that probucol could preserve endothelial function by enhancing prostacyclin generation [42], reducing the level of endogenous nitric oxide (NO) synthase inhibitor [43], and inhibiting the expression of various adhesion molecules [44]. Probucol could also promote proliferation [42] and reduce apoptosis of endothelial cells that induced by oxidative injury [45-47]. Impaired ischemia-induced neovascularization by cigarette smoke could also be rescued by probucol via improved function of EPCs [48]. Probucol treatment could also protect EPCs from oxidized high-density lipoprotein (ox-HDL)-induced apoptosis [49]. The present study aimed to determine whether probucol could have protective effects on circulating MNCs and EPCs in mouse treated with ox-LDL and in hyperlipidemic patients with elevated ox-LDL level. We observed that probucol could rescue the decreased population of circulating MNCs and EPCs by ox-LDL in mouse and in hyperlipidemic patients with increased level of ox-LDL.

Materials and Methods

Ox-LDL preparation

Following Institutional Review Board approval and obtaining informed consent, blood samples were collected in heparinized tubes from healthy volunteers for the study. Plasma was obtained with centrifugation

at 1500g for 20 min. Lipoproteins were isolated from the plasma by sequential ultracentrifugation using a Beckman TL-100 tabletop ultracentrifuge (Beckman, Palo Alto, CA) as described [50]. The isolated lipoproteins were dialyzed against 0.3mM EDTA in 1× phosphate-buffered saline (PBS, pH 7.4) overnight and subsequently filter-sterilized with 0.22 µm filter. The protein concentration in the lipoproteins was determined using the Lowry's method. Ox-LDL was produced from the native LDL immediately after dialysis using 5 µM copper sulphate, and was stopped by adding EDTA (0.25 mM, final concentration) as described [51].

Animal models

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health (NIH Publication no. 85-23, revised 1996). The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Ohio State University Wexner Medical Center, Columbus, OH, USA. Twenty-four wild type male C57 BL/6 mice (4-6 weeks old, Jackson Lab, ME, USA) were divided randomly into control group, ox-LDL treatment group, and probucol group. The mice in ox-LDL treatment group and probucol group were given 50µg ox-LDL via tail vein injection (once daily for 3 days), while the mice in control group were given equal volume of PBS (once daily for 3 days). For the probucol group, probucol was first dissolved in 99% ethanol and then diluted in PBS. The prepared probucol was used to pre-treat the mice (500 mg/kg/d) 24 hours before ox-LDL injection and daily for 3 days with ox-LDL treatment via intragastric administration.

Intracellular and extracellular ROS detection

Blood was harvested from the mice after intravenous injection with PBS (control) or ox-LDL (50 µg) with or without probucol treatment for 3 days. Red blood cells (RBC) were eliminated using RBC lysis as described [36]. The blood intracellular ROS formation was determined as described [36]. Plasma and BM fluid were collected for extracellular ROS detection using electron paramagnetic resonance (EPR) as described [36].

Murine MNCs, EPCs and human EPCs analysis

After collection of BM and blood cells, MNCs were isolated as described [52]. Cell surface markers CD34, Flk-1 and KDR were used to identify EPCs. The cells positive for CD34⁺/Flk-1⁺ were identified as EPCs for mice, and the cells positive for CD34⁺/KDR⁺ were considered as EPCs for patients. Mouse Flk-1 APC-Cy7 and mouse CD34 FITC were obtained from Becton Dickinson Biosciences (NJ, USA) and eBioscience (San Diego, CA, USA) respectively, while the other antibodies were purchase from Biolegend (San Diego, CA, USA). After elimination of RBC, flow cytometry analysis was performed to identify EPCs using the LSRII system (Becton Dickinson Biosciences, NJ, USA).

Patient selection and human ox-LDL measurement

The patient study was conducted at the Shandong Provincial Hospital, Shandong University, Jinan, China. The protocol was reviewed and approved by the university ethical review board. All patients provided their written informed consent. A total of 10 patients who had CAD and hyperlipidemia with age of at least 21 years old were recruited into the study. Age- and sex-matched healthy volunteers were recruited as the control. Patients were randomly divided into 2 groups with 5 patients in each group: probucol treatment group and placebo control. Age-matched healthy volunteers were recruited as healthy control. Baseline fasting lipid profile, serum ox-LDL level, blood glucose, C-reactive protein (CRP), superoxide dismutase (SOD), thyroid stimulating hormone (TSH), kidney and liver functions were obtained from all the patients. Patients in the probucol treatment group received 500 mg probucol twice a day orally for 7 days with no further treatment afterwards, while the patients in the control group were given placebo. The patients and the treating physicians had no knowledge on what they received (double blind). After one week of treatment and one week after discontinuing probucol, blood samples were collected to determine the fasting lipid profile, serum ox-LDL level, blood glucose, CRP, SOD, TSH, kidney and liver functions, as well as the populations of circulating MNCs and EPCs. The patients' lipid profile was determined using an ARCHITECT ci16200 Integrated System (Abbott, Illinois, US) and an electrochemiluminescent procedure (Cobas E601; Roche, Basel, Switzerland). Patients' plasma ox-LDL was measured using human ox-LDL ELISA kit (Blue Gene, Shanghai, China).

Statistical analysis

All the data were presented as means $\pm$ standard deviation (SD), and statistically analyzed using unpaired Student t-test (two-sided) for two groups of data or two way ANOVA (analysis of variance) (PRISM Version 5.0.; GraphPad Software, Inc., San Diego, CA) followed by post hoc conservative Bonferroni's test for three groups of data to minimize type I error as appropriate. Normal distribution of data was tested using the Shapiro-Wilk *W*-test, and equal variance was tested using the *F*-test. When the null hypothesis of normality and/or equal variance was rejected, the non-parametric Mann-Whitney *U*-test was used. The differences were considered statistically significant when a two-tailed $p < 0.05$.

Result

Probucol prevented ox-LDL-mediated reduction of BM mononuclear cells and circulating EPCs

To determine the effects of ox-LDL on EPCs, we first injected 50 μ g human ox-LDL into wild-type (WT) mice for 3 days. The populations of BM mononuclear cells (MNCs) and circulating EPCs were significantly decreased in the mice with ox-LDL treatment (Fig.1A and B). To investigate the protective effect of probucol, we pre-treated the mice with probucol prior to the injection of ox-LDL. Probucol effectively prevented ox-LDL-induced reduction in the population of circulating EPCs and BM MNCs.

Probucol attenuated both intracellular and extracellular ROS production

To explore the mechanisms for the action of ox-LDL on circulating EPCs and BM MNCs, we measured both intracellular and extracellular ROS production in the blood and BM.

Fig. 1. Probucol rescued the circulating MNCs and EPCs in mouse with ox-LDL treatment. After 3 days of ox-LDL treatment, the circulating MNCs (A) and EPCs (B) were significantly decreased. The decreased EPCs were normalized following probucol treatment. PBS: C57BL/6 mouse with PBS treatment; OX: C57BL/6 mouse with ox-LDL injection; OX+Prob: C57BL/6 mouse with probucol treatment following ox-LDL injection; Mono: mouse mononuclear cell; M CD34⁺/Flk-1⁺: mouse CD34⁺/Flk-1⁺ cell; SSC-A: side scatter analysis; FSC-A: forward scatter analysis. * PBS vs OX, $p < 0.05$, $n = 8$; # ox vs ox+prob, $p < 0.05$, $n = 8$.

Fig. 2. Ox-LDL-induced extracellular ROS production was completely blocked by probucol. Both BM (A) and blood (B) extracellular ROS levels were significantly increased after ox-LDL injection. Probucol treatment effectively blocked the ROS generation. BM EPR intensity: bone marrow EPR intensity; BL EPR intensity: blood EPR intensity. * PBS vs OX, $p < 0.05$, $n = 8$; # ox vs ox+prob, $p < 0.05$, $n = 8$.

Fig. 3. Ox-LDL-induced blood intracellular ROS production was prevented with probucol treatment. After ox-LDL injection, the blood intracellular ROS production was significantly increased. Probucol effectively blocked the ROS production from ox-LDL. There was no extra ROS production in BM cells with ox-LDL treatment. * PBS vs OX, $p < 0.05$, $n = 8$; # ox vs ox+prob, $p < 0.05$, $n = 8$.

Treatment of the mice with ox-LDL significantly increased the extracellular ROS in murine BM and blood cells over 2 folds compared to the control (Fig. 2A and B). The murine blood intracellular ROS level was also significantly elevated in the mice treated with ox-LDL compared to the mice with PBS treatment (Fig. 3B), while there was no change in murine

Table 1. Human plasma lipid profile. Plasma lipid profile was determined in the patients with coronary artery disease and hyperlipidemia with and without probucol treatment. Baseline: Patient without placebo or probucol treatment; Treatment: Patient with placebo or probucol treatment for 7 days; Post treatment: patient stopped treating placebo or probucol for 7 days. HDL: high density lipoprotein; LDL: low density lipoprotein; TRG: triglycerides; TC: total cholesterol

		HDL(mmol/L)	LDL(mmol/L)	TG(mmol/L)	TC(mmol/L)
Baseline	Placebo (Mean $\pm$ SD, n=5)	1.10 $\pm$ 0.24	2.88 $\pm$ 0.54	1.70 $\pm$ 0.14	4.85 $\pm$ 0.92
	Prob (Mean $\pm$ SD, n=5)	0.96 $\pm$ 0.14	2.91 $\pm$ 0.62	2.07 $\pm$ 0.84	4.80 $\pm$ 0.92
Treatment	Placebo (Mean $\pm$ SD, n=5)	1.25 $\pm$ 0.22	2.57 $\pm$ 0.68	1.74 $\pm$ 0.15	4.92 $\pm$ 0.81
	Prob (Mean $\pm$ SD, n=5)	0.85 $\pm$ 0.22	2.27 $\pm$ 0.77	2.29 $\pm$ 0.58	4.00 $\pm$ 1.51
Post treatment	Placebo (Mean $\pm$ SD, n=5)	1.25 $\pm$ 0.21	2.81 $\pm$ 0.72	1.55 $\pm$ 0.14	5.14 $\pm$ 0.62
	Prob (Mean $\pm$ SD, n=5)	0.98 $\pm$ 0.25	2.46 $\pm$ 0.87	1.72 $\pm$ 0.58	4.07 $\pm$ 1.39

BM intracellular ROS level (Fig. 3A). Pre-treatment with probucol effectively blocked the production of extracellular ROS in the BM and blood as well as intracellular blood ROS production in the mice treated with ox-LDL as shown in Fig. 2 and Fig. 3.

Probucol treatment decreased serum ox-LDL level and rescued circulating EPCs in hyperlipidemic patients

To study the effect of probucol on ox-LDL and EPCs in human, we measured the plasma lipid profile in patients with hyperlipidemia with and without probucol treatment. LDL, non-HDL lipoprotein, and ox-LDL were all significantly increased in the patients with hyperlipidemia over the age- and sex-matched healthy individuals (controls) as expected (Table 1). After one week of probucol treatment, there were no significant changes in serum LDL, non-HDL lipoprotein levels, blood glucose, TSH, kidney and liver functions in the patients (data not shown). However, the serum ox-LDL was significantly decreased in the patients with probucol treatment as compared with the placebo group. Decreased level of serum ox-LDL was still present in the patients one week after discontinuation of probucol (Fig. 4). In parallel to decreased serum ox-LDL level, the level of circulating EPCs was partially and yet significantly rescued in the patients with probucol treatment compared with the placebo group. The level of EPCs maintained at a higher level in the patients one week after probucol discontinuation as shown in Fig. 5.

Probucol increased plasma SOD and decreased CRP levels in hyperlipidemic patients

Since SOD and CRP are associated with ROS production, we measured plasma SOD and CRP levels in the hyperlipidemic patients with and without probucol treatment. The plasma SOD was significantly increased while CRP was significantly reduced in the patients with one week of probucol treatment as compared with the placebo group. The effect of probucol on SOD and CRP persisted in the patients one week after discontinuation of probucol treatment (Fig. 6).

Discussion

In the present study, we demonstrated that probucol reversed the effects of ox-LDL on circulating MNCs and EPCs, and effectively prevented ox-LDL-induced increase in BM and

Fig. 4. Probucol treatment decreased plasma ox-LDL level in hyperlipidemic patients. Plasma ox-LDL level was measured using ELISA in the patients with documented coronary artery disease (CAD) and hyperlipidemia with and without probucol treatment. The serum ox-LDL level was significantly elevated in the patients with hyperlipidemia as compared with the healthy volunteers. Probucol treatment significantly decreased the serum ox-LDL level in the patients. The serum ox-LDL remained at the decreased level in the patients one week after discontinuation of probucol. Healthy Ctrl: healthy volunteer as control group. Placebo: hyperlipidemic patients with placebo treatment. Prob: hyperlipidemic patients with probucol treatment. * placebo treatment vs healthy control, $P < 0.05$, $n = 5$; ** probucol treatment vs healthy control, $p < 0.05$, $n = 5$; # probucol treatment vs placebo, $p < 0.05$, $n = 5$.

Fig. 5. Probucol treatment partially rescued EPCs in hyperlipidemic patients. The EPCs level was significantly decreased in patients with hyperlipidemia, while the declined EPCs were partially rescued with probucol treatment and post treatment for 1 week respectively. * placebo treatment vs healthy control, $P < 0.001$, $n = 5$; ** probucol treatment vs healthy control, $p < 0.001$, $n = 5$; # probucol treatment vs placebo, $p < 0.001$, $n = 5$.

blood extracellular ROS and blood intracellular ROS levels in mouse. Probucol treatment partially rescued the diminished EPCs level in patients with hyperlipidemia in association with reduction of ox-LDL and CRP and increase in SOD level.

It has been shown that ox-LDL significantly impairs the function, survival and proliferation of stem cells and progenitor cells [53]. Ox-LDL induces senescence [54], accelerates the ageing of hematopoietic stem cells *in vivo* [55], and inhibits their proliferation, migration, adhesion and differentiation [17, 30, 53, 56, 57]. Previous studies showed that the number of circulating EPCs was inversely correlated with total cholesterol, LDL-cholesterol and ox-LDL levels [11, 58-60]. However, Zhang et al. reported that the transmigration rates of BM mesenchymal stem cells (MSCs) and cell-cell adhesion between BM MSCs and MCs were significantly increased with ox-LDL treatment in a dose- and time-dependent manner [61]. Our previous [36] and present studies demonstrated that ox-LDL could significantly decrease both circulating MNCs and EPCs levels.

Ox-LDL significantly decreased the proliferation, migration, and adhesion capacity of EPCs via increased ROS formation [62]. It was reported that a significant amount of ROS was produced spontaneously from ox-LDL *in vitro* and *in vivo* [53, 63]. ROS and oxidative

Fig. 6. Probucol treatment decreased plasma CRP and increased SOD level in hyperlipidemic patients. The plasma SOD level was significantly decreased and CRP was notably increased in patients with hyperlipidemia. Probucol partially reversed the effects of hyperlipidemia on plasma SOD and CRP in the patients with hyperlipidemia. SOD: superoxide dismutase. CRP: C-reactive protein. * placebo treatment vs healthy control, $P < 0.05$, $n = 5$; ** probucol treatment vs healthy control, $p < 0.05$, $n = 5$; # probucol treatment vs placebo, $p < 0.05$, $n = 5$.

stress were involved in EPC dysfunction in many diseases including hyperlipidemia, diabetes, coronary artery disease, renal ischemia-reperfusion injury and air pollution related cardiovascular disease [64-67]. Our data showed that ox-LDL was able to increase both BM and blood extracellular and blood intracellular ROS levels in mouse with ox-LDL treatment. In addition, ox-LDL and hyperlipidemia-induced reduction in EPCs in BM and blood were associated with ROS production [36]. On the other hand, antioxidant enzyme SOD could attenuate ROS generation *in vivo* and *in vitro* [36], thus SOD deficiency leads to increased ROS production and endothelial dysfunction during nitroglycerin tolerance [37]. In addition, plasma CRP is known as a pro-inflammatory marker [38], and is associated with ROS generation [39] and SOD activities [40]. Therefore, we used SOD and CRP as an indirect evidence for ROS production in the patient study. We found that the SOD was significantly decreased while CRP was increased in patients with hyperlipidemia.

Several studies reviewed the protective reagents and methods for EPCs including tripterine, urinary trypsin inhibitor, microRNA-130a, interruption of CD40 pathway, and GM-CSF [68-71]. As a cholesterol-lowering medicine originally, probucol significantly inhibits the initiation and development of atherosclerosis via multiple mechanisms including suppressing ROS formation [72-74], rescuing endothelial function [75-77], inhibiting the activation and adhesion of monocytes [78], attenuating growth and migration of VSMCs [76, 79, 80], altering proliferation and apoptosis of VSMCs [80-82] and macrophages [83], as well as decreasing cytokine secretion in macrophages of [84]. Probucol prevents LDL oxidation [85, 86], increases SOD level and activity [87], decreases the levels of ox-LDL and CRP [72]. Probucol has been reported to rescue cigarette smoke-induced impairment of ischemia-induced neovascularization by improving the function of EPCs [48], and protect EPCs from oxidized high-density lipoprotein (ox-HDL) [49]. Additionally, it has been reported that probucol is able to prevent ROS-induced inactivation of endothelium-derived NO [88], reduce endogenous NO synthase inhibitor [43], and increase the level and function of NO, which could regulate the self-renewal, viability, migration, proliferation, and differentiation of EPCs [89]. In the present study, we observed that probucol effectively preserved the populations of circulating MNCs and EPCs and blocked ROS formation in the mice with ox-LDL treatment. In the human study, EPCs were partially rescued in association with decreased levels of ox-LDL and CRP and increased level of SOD following probucol treatment in the patients with hyperlipidemia.

Fig. 7. Possible mechanisms on the beneficial effect of probucol on circulating EPCs in hyperlipidemia. Ox-LDL and CRP levels were increased, and SOD was decreased in the hyperlipidemic state *in vivo*. A significant amount of ROS was produced with elevated ox-LDL and CRP level and decreased SOD levels *in vivo* which could contribute to the decreased level of EPCs. Probucol decreased the levels of ox-LDL and CRP, increased the level of SOD, leading to decreased ROS generation. Probucol as an antioxidant could also inhibit ROS production directly. The combined outcome of the protective effects of probucol could normalize the population of EPCs that was decreased with ox-LDL treatment or in hyperlipidemia. -: diminish; ↓: decrease; ↑: increase.

The mechanisms for the protective effects of probucol on ox-LDL-mediated reduction of EPCs are very complex, and many mechanisms are still unknown. Although our present study showed that probucol rescued EPCs through inhibition of ROS production from ox-LDL, there are many questions that need to be addressed. For example, does probucol also protect BM stem cells which are the main source of EPCs? Does probucol affect the proliferation and/or apoptosis of EPCs? Can probucol restore the function of EPCs impaired by ox-LDL? Does ox-LDL trigger autophagy of EPCs? If so, can probucol prevent this process? All these questions require further studies.

In conclusion, data from the present study demonstrated that probucol effectively reversed the effects of ox-LDL on circulating MNCs and EPCs in mouse via inhibition of BM and blood extracellular ROS and blood intracellular ROS production. For human study, probucol partially rescued the diminished EPCs level in hyperlipidemic patients in association with reduction of ox-LDL and CRP and increase in SOD level (Fig. 7).

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Disclosure Statement

None.

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