Research Article

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Salvianolic acid B inhibits low-density lipoprotein oxidation and neointimal hyperplasia in endothelium-denuded hypercholesterolaemic rabbits

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Abstract

BACKGROUND: Atherosclerosis and restenosis are inflammatory responses involving free radicals and lipid peroxidation and may be prevented/cured by antioxidant-mediated lipid peroxidation inhibition. Salvianolic acid (Sal B), a water-soluble antioxidant obtained from a Chinese medicinal herb, is believed to have multiple preventive and therapeutic effects against human vascular diseases. In this study the *in vitro* and *in vivo* inhibitory effects of Sal B on oxidative stress were determined.

RESULTS: In human aortic endothelial cells (HAECs), Sal B reduced oxidative stress, inhibited low-density lipoprotein (LDL) oxidation and reduced oxidised LDL-induced cytotoxicity. Sal B inhibited Cu^{2+}-induced LDL oxidation *in vitro* (with a potency 16.3 times that of probucol) and attenuated HAEC-mediated LDL oxidation as well as reactive oxygen species (ROS) production. In cholesterol-fed New Zealand White rabbits (with probucol as positive control), Sal B intake reduced Cu^{2+}-induced LDL oxidation, lipid deposition in the thoracic aorta, intimal thickness of the aortic arch and thoracic aorta and neointimal formation in the abdominal aorta.

CONCLUSION: The data obtained in this study suggest that Sal B protects HAECs from oxidative injury-mediated cell death via inhibition of ROS production. The antioxidant activity of Sal B may help explain its efficacy in the treatment of vascular diseases.

Keywords: salvianolic acid B; oxidised low-density lipoprotein; endothelial cells; atherosclerosis; restenosis

INTRODUCTION

Endothelial dysfunction, blood vessel inflammation and smooth muscle cell proliferation are characteristic events in atherosclerosis and cardiovascular diseases. Reactive oxygen species (ROS) stimulate cell injury and death by oxidative modification of proteins, carbohydrates, lipids and DNA.[1–3] Intracellular and extracellular oxidative stress can induce oxidative modification of native low-density lipoprotein (LDL) to oxLDL. Recent studies have demonstrated that oxLDL plays an important role in the initiation and progression of atherosclerosis.[4] OxLDL can damage endothelial cells and is involved in endothelial dysfunction via several different mechanisms.[5] OxLDL induces up-regulation of adhesion molecules, cytokines and chemokines and can cause necrosis in endothelial cells.[1] OxLDL has also been shown to increase intracellular ROS generation, so antioxidant compounds may reduce atherogenesis and improve vascular function via inhibition of LDL oxidative modification.[6–8]

The herb *Salvia miltiorrhiza* Bunge is often used in folk medicine in China, Japan and Taiwan for the treatment of cardiovascular disorders (called blood stasis in traditional Chinese medicine).[9] *Salvia miltiorrhiza* aqueous ethanol extract (SME) inhibits LDL oxidation *in vitro* and *ex vivo* and reduces atherosclerosis in hypercholesterolaemic rabbits.[10] The major water-soluble constituents

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of *S. miltiorrhiza* are danshensu, lithospermic acid, protocatechuic aldehyde, rosemarinic acid, salvianolic acid A (Sal A) and Sal B.[1,11–13] Sal B is the most bioactive polyphenolic component of the water-soluble fraction of *S. miltiorrhiza*.[14–16] The main pharmacological activities of Sal B include inhibition of lipopolysaccharide (LPS)-inducible COX-2 in human aortic smooth muscle cells (HASMCs) via JNK and ERK suppression,[17] reduction of MMP-2 and MMP-9 in cholesterol-fed mice and LPS-stimulated HASMCs,[18] reduction of leucocyte adhesion to human aortic endothelial cells (HAECs)[19] and attenuation of ischaemia–reperfusion injury.[20,21] Additionally, administration of Sal B resulted in an improvement in regional cerebral blood flow in the ischaemic cerebral hemisphere in rats,[20,22] attenuation of apoptosis in rat cortical neurons[23] and neuroprotection in cerebral ischaemia.[24] Furthermore, Sal B has also been shown to protect endothelial cells from oxidative stress,[25] to inhibit TGF-β1-induced myofibroblast transition and restore epithelial morphology[26] and to inhibit platelet aggregation and adhesion under flow conditions.[27]

The majority of studies have attributed the cellular activities of Sal B to the antioxidant activity of this molecule. Our previous study showed that the Sal B-rich fraction of SME inhibits LDL oxidation both *in vitro* and *ex vivo* and can reduce atherosclerosis in cholesterol-fed rabbits.[10] The effects of pure Sal B on HAECs and hypercholesterolaemic rabbits are still unclear. In this paper we describe the effects of Sal B on HAECs treated with oxLDL and on atherosclerotic lesions and neointimal formation after balloon injury of the aorta in cholesterol-fed rabbits.

MATERIALS AND METHODS

Reagents

Probucol was obtained from Sigma (St. Louis, MO, USA) and dissolved in ethanol as a 10 mmol L^{-1} stock solution. Sal B was purified as described previously.[19] In brief, dry roots of *S. miltiorrhiza* were ground to a powder, which was then extracted with ten volumes of a 4 : 1 (vol/vol) mixture of water and ethanol at room temperature for 24 h. After paper filtration of the mixture, the solvent was evaporated under reduced pressure and the solid material (SME) was stored at −70 °C. The concentration of Sal B in the SME, determined by reverse phase high-performance liquid chromatography, was 44 g kg^{-1}. Since Sal B is water-soluble and has low pK_a values similar to those of carboxylic acids, the content of Sal B was further increased by adjustment of the pH of the concentrated SME and precipitation with acetone/water. After ion exchange and Sephadex LH-20 column chromatography, Sal B was obtained at >980 g kg^{-1} purity. For use, it was dissolved as a 14 mmol L^{-1} stock solution in phosphate-buffered saline (PBS, pH 7.4). Unless specified otherwise, all reagents were from Sigma.

Culture of HAECs

HAECs were provided as cryopreserved tertiary cultures by Cascade Biologics (Portland, OR, USA) and were grown in culture flasks in endothelial cell growth medium (Cascade Biologics) supplemented with foetal bovine serum (20 mL L^{-1}), human epidermal growth factor (10 ng mL^{-1}), human fibroblast growth factor (3 ng mL^{-1}), heparin (10 µg mL^{-1}), penicillin (100 U mL^{-1}), streptomycin (100 pg mL^{-1}) and Fungizone (1.25 µg mL^{-1}). The growth medium was changed every other day until confluence. Cells under passage 8 were used for this study. The purity of HAEC cultures was verified by staining with monoclonal antibody against von Willebrand factor.

LDL isolation, oxLDL preparation and measurement of inhibition of LDL oxidation

Fasting plasma samples were collected from healthy male adults not given vitamin supplements. LDL was obtained by ultracentrifugation in the density range $1.019 < d < 1.063$ (adjusted with NaBr)[6] and extensively dialysed against PBS (5 mmol L^{-1} phosphate buffer and 145 mmol L^{-1} NaCl, pH 7.4) at 4 °C under N_2 for 24 h. After dialysis, negative LDL was stored at 4 °C under N_2 in the dark.

OxLDL was prepared by incubating LDL (1 mg mL^{-1}) with $CuSO_4$ (10 µmol L^{-1} final concentration) at 37 °C for 18 h. After incubation, 150 mL of ethylene diamine tetraacetic acid (EDTA, 2 mmol L^{-1}) was added, the mixture was dialysed against PBS at 4 °C for 6 h to remove EDTA and the resulting oxLDL was stored at 4 °C under N_2. Formation of malondialdehyde (MDA) was determined as thiobarbituric acid-reactive substances (TBARS).[28,29] In brief, an aliquot of incubation solution was mixed with 1,1,3,3-tetramethoxypropane solution (6 g L^{-1}) and heated at 95 °C for 45 min. After centrifugation, TBARS in the supernatant were measured by the absorbance at 532 nm.

LDL oxidation was also carried out by first incubating various concentrations of Sal B or probucol with 10 µL of LDL (1 mg mL^{-1}). Then $CuSO_4$ was added (10 µmol L^{-1} final concentration) and the mixture was incubated at 37 °C for 90 min *in vitro*. The optical density (OD) at 234 nm was read to determine the production of conjugated diene. The antioxidant power of Sal B and probucol was calculated as a percentage compared with the control (Cu^{2+} alone).

Measurement of cell-mediated LDL oxidation

HAECs were plated at a density of 2×10^4 cells per well in 96-well plates and allowed to incubate for 16 h. Subsequently, 100 µL aliquots of medium containing various concentrations of Sal B or probucol and 100 µg mL^{-1} LDL were added. After 24 h of incubation the medium was carefully discarded and the volume of oxLDL was determined by TBARS measurement.[30]

Measurement of cytotoxicity

HAECs were plated at a density of 2×10^4 cells per well in 96-well plates and treated with various concentrations of Sal B or probucol combined with 30 µg mL^{-1} oxLDL. After 24 h of incubation, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, MTT (0.5 mg mL^{-1}) was applied to the cells for 4 h to allow the conversion of MTT into formazan crystals, then, after washing with PBS, the cells were lysed with dimethyl sulfoxide and the absorbance at 530 and 690 nm was read with a DIAS microplate reader (Dynex Technologies, Chantilly, VA, USA). The changes in OD caused by cytokine and drug treatment were used as an index of cell viability, normalised to cells incubated in control medium, which were considered 100% viable.

Assessment of intracellular ROS production

The effect of Sal B on ROS production in HAECs was determined by a fluorometric assay using 10 µmol L^{-1} 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) as the probe. Confluent HAECs in 48-well plates were pretreated for 18 h with various concentrations of Sal B or probucol, then oxLDL (30 µg mL^{-1}) was added for 1 h. The fluorescence intensity (relative fluorescence units) was measured at 485 nm excitation and 530 nm emission using a fluorescence microplate reader. Cells were also viewed and photographed under a fluorescent microscope.

Animal care and experimental procedures

A total of 30 New Zealand White rabbits of body weight (BW) 2.5–3 kg were used for this study. The experimental procedures, animal care and handling were carried out according to the 'Guide for the care and use of laboratory animals' published by the US National Institute of Health. After 1 week on a commercial rabbit chow diet (Purina Mills, Gray Summit, MO, USA), 27 of the animals were randomly allocated to one of three groups: (1) a 20 g kg^{-1} high-cholesterol-fed (C) group, (2) a cholesterol-fed and probucol-treated (P) group, with oral ingestion of probucol (5 g kg^{-1} BW day^{-1}), and (3) a cholesterol-fed, endothelium-denuded and Sal B-treated (Sal B) group, with oral ingestion of Sal B (1.5 g kg^{-1} BW day^{-1}). The selection of Sal B dose was based on its antioxidant activity compared with that of probucol in Cu^{2+}-induced LDL oxidation. Water was allowed *ad libitum*. The animals were bled periodically for the assessment of plasma cholesterol, liver function and renal function. Three weeks after starting the above diets, the animals were anaesthetised with intramuscular xylazine (5 mg kg^{-1}) and ketamine hydrochloride (35 mg kg^{-1}). Surgery was performed under sterile conditions. The right femoral artery was exposed through an incision line 1.5–2 cm below the inguinal ligament, and an arterial embolectomy catheter (Baxter Healthcare, High Wycombe, UK) was introduced retrogradely into the lower abdominal aorta for 16 cm, measured from the tip of the catheter. Denudation was then performed by inflating a balloon until resistance was felt. This procedure was repeated three times. The surgical wound was closed and the animals were continued on a high-cholesterol diet with different antioxidant treatments.

The remaining three rabbits were fed control chow (normal diet) for 6 weeks and used as the normal control group. At the end of the sixth week of the experiment, all rabbits were euthanised by intravenous injection of 35–40 mg kg^{-1} sodium pentobarbital and sacrificed. The aortic arches, thoracic aortas and abdominal aortas were removed, gently dissected free of adhering tissue and then rinsed with ice-cold PBS. A segment of each aorta was immersion fixed with 40 g L^{-1} paraformaldehyde for light microscopy.

Biochemical measurement

Blood samples were collected after 0, 2, 4 and 6 weeks of feeding. The samples were centrifuged and the plasma was stored at −20 °C prior to analysis. Plasma levels of total cholesterol, triglyceride, blood urea nitrogen (BUN), creatinine, glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were measured using reagent strips of a fully automated dry chemistry system.

Isolation of LDL and measurement of inhibition of LDL oxidation

Fasting blood samples were collected into evacuated tubes (K_3-EDTA) from rabbits after 3, 4 and 6 weeks of feeding. The LDL fraction ($1.019 < d < 1.063$) was obtained by ultracentrifugation and extensively dialysed against PBS (pH 7.4). The dialysed LDL (0.06 mg mL^{-1}) was incubated with $CuSO_4$ (5 μmol L^{-1}) at 37 °C to induce oxidation. The concentration of conjugated diene was determined by the UV absorption at 232 nm.

Determination of extent of atherosclerosis

The thoracic aortas were stained with Sudan IV solution to visualise the atherosclerotic lesion area. Sudanophilic areas were photographed and quantitatively measured by LV-2 computer-assisted planimetry. The extent of lesions was expressed as the proportion of the total surface area with sudanophilic area.

Haematoxylin–eosin staining and morphometry

The aortic arches, thoracic aortas and four arterial segments cut from the abdominal aortas were immersion fixed in 40 g L^{-1} buffered paraformaldehyde at 4 °C for 3 h and subsequently dehydrated in sequential 50, 70, 80, 90 and 100% (v/v) alcohol washes, cleared in xylene and embedded in paraffin. Serial cross-sectioning of each arterial segment was performed. Five cross-sections of 5 μm thickness were randomly selected from each segment and stained with haematoxylin and eosin (H&E). Morphometric analysis of the 20 arterial cross-sections per animal was performed using an LV-2 image analyser (Window Instruments, Taipei, Taiwan).[31] For each arterial cross-section specimen the intimal thickness was measured at eight equally spaced points. The intimal thicknesss of each cross-section was calculated as the mean of these eight values.

Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). Differences in mean values among different groups were determined by one-way analysis of variance followed by the *post hoc* Dunnett test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Sal B inhibition of *in vitro* LDL oxidation

LDL is modified via different mechanisms, including cell- and Cu^{2+}-mediated oxidation.[5] Our previous study demonstrated that LDL from SME-treated rabbits was more resistant to Cu^{2+}-induced oxidation.[10] Here we examined the *in vitro* effect of Sal B on Cu^{2+}- and cell-mediated LDL oxidation. Figure 1A shows that both Sal B and probucol (a lipid-lowering drug with antioxidant properties, used in this study as a positive control for antioxidant activity) inhibited Cu^{2+}-induced LDL oxidation, with IC_{50} values (obtained from the concentration–response curves in Fig. 1A) of 0.17 and 2.77 μg mL^{-1} respectively. Based on the IC_{50} values, the antioxidant potency of Sal B was about 16.3 times that of probucol.

The extent of LDL oxidation after incubation with endothelial cells was determined from the amount of TBARS generated in the cell culture medium.[30] Sal B (1.25, 2.5, 5 or 10 μmol L^{-1}) inhibited endothelial cell-induced LDL oxidation and significantly reduced TBARS levels in a dose-dependent manner ($P < 0.05$) (Fig. 1B).

Sal B inhibition of oxLDL-induced cytotoxicity and ROS production in endothelial cells

To observe the effects of Sal B and probucol on the viability of oxLDL-treated HAECs, the MTT assay was performed. The results indicated that cell viability was unaffected by 80 μmol L^{-1} Sal B (data not shown). The viability of cells treated with 30 μg mL^{-1} oxLDL alone was 20% of that of control cells. Treatment of cells with 30 μg mL^{-1} oxLDL combined with Sal B increased cell viability. Cells treated with 5 μmol L^{-1} Sal B showed a viability of 58 ± 3% compared with control cells, a significant increase ($P < 0.05$). The viability of cells treated with 30 μg mL^{-1} oxLDL combined with 2.5 or 5 μmol L^{-1} probucol also increased significantly (75 ± 7 and 93 ± 9% respectively, $P < 0.05$ for both concentrations) compared with cells treated with oxLDL alone (Fig. 2).

HAECs were pretreated with 1.25, 2.5, 5 or 10 μmol L^{-1} probucol or Sal B for 18 h and interacted with DCFH-DA, then stimulated with 30 μg mL^{-1} oxLDL for 1 h. ROS levels induced in endothelial cells treated with 10 μmol L^{-1} Sal B for 18 h were significantly decreased

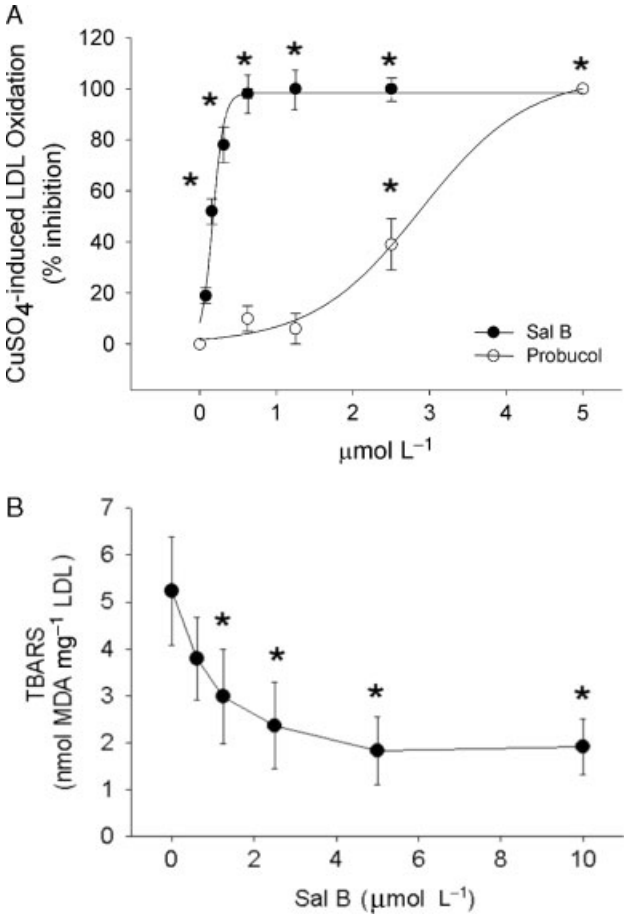

Figure 1. Effect of Sal B on inhibition of LDL oxidation. (A) Sal B inhibition of LDL oxidation induced by $CuSO_4$. (B) Cells were incubated with various concentrations of Sal B and 100 μg mL^{-1} LDL. After 24 h of incubation the volume of oxLDL was determined by TBARS measurement. Sal B efficiently reduced LDL oxidation induced by human aortic endothelial cells. *$P < 0.05$ compared with control.

compared with the control, with a 47.9 ± 6.7% inhibition effect (Fig. 3A). As shown in Fig. 3B, fluorescence density was high in cells treated with 30 μg mL^{-1} oxLDL for 1 h. This effect was reduced by pretreatment with Sal B, reflecting decreased intracellular ROS production (Fig. 3B).

Effects of Sal B treatment on biochemical parameters in plasma of cholesterol-fed rabbits

Over the experimental period, there were no differences in weight gain and final weight among the three groups of control, Sal B- and probucol-treated rabbits fed a high-cholesterol diet. There were also no significant differences in plasma levels of BUN, creatinine or triglyceride. However, plasma levels of total GOT, GPT and cholesterol showed a gradual increase during the experimental period and a significant increase towards the end. Sal B treatment significantly reduced GOT and GPT concentrations at week 6 but did not affect cholesterol concentration (Table 1).

Sal B reduction of LDL oxidation susceptibility in rabbits

Next, LDLs isolated from hypercholesterolaemic rabbits in each diet group at weeks 3, 4 and 6 were examined for oxidation. LDLs collected from the Sal B- and probucol-treated groups were more resistant to oxidation than those from the high-cholesterol control

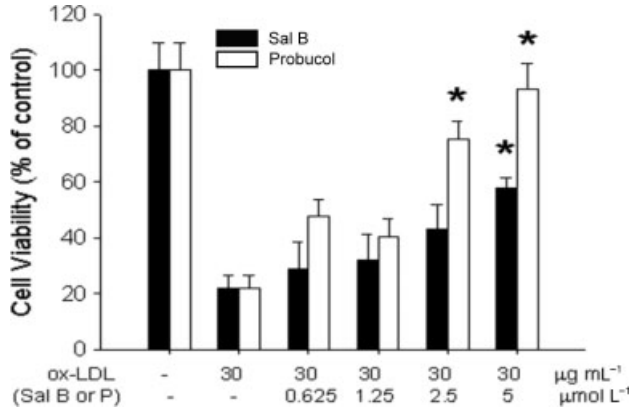

Figure 2. Effect of Sal B and probucol on oxLDL-induced cytotoxicity. HAECs were treated with the indicated concentrations of probucol or Sal B followed by 30 μg mL^{-1} oxLDL for 24 h. Cell viability was measured by MTT assay. Cell viability was increased in HAECs treated with 2.5 or 5 μmol L^{-1} Sal B or 5 μmol L^{-1} probucol. *$P < 0.05$ compared with oxLDL group.

group. The time required to achieve half-maximum oxidation ($T_{1/2}$) was defined as the antioxidativity. After 6 weeks the antioxidativity of the high-cholesterol group was significantly decreased. The antioxidativity of both the probucol- and Sal B-treated groups was significantly higher than that of the high-cholesterol group at weeks 4 and 6 (Fig. 4).

Sal B reduction of atheroma area in thoracic aorta of cholesterol-fed rabbits

After 6 weeks on individual diets, thoracic aortas were obtained and stained with Sudan IV (Fig. 5). In the normal group, no atheroma was found in the thoracic aorta. The average atheroma areas of the thoracic aorta in the cholesterol diet, cholesterol diet plus probucol and cholesterol diet plus Sal B groups were 0.120 ± 0.026, 0.028 ± 0.007 and 0.048 ± 0.008 respectively. The atheroma areas in the cholesterol diet plus Sal B and cholesterol diet plus probucol groups were significantly smaller than those in the cholesterol diet group ($P < 0.05$).

Sal B reduction of intimal area in aortic arch and thoracic aorta of cholesterol-fed rabbits

The intima in the aortic arch and thoracic aorta in the cholesterol diet group was thicker than that in the cholesterol diet plus probucol group. The thickness of the intima in the thoracic aorta and aortic arch in the cholesterol diet plus Sal B group was identical to that in the cholesterol diet plus probucol group.

The ratio of intimal thickness in the aortic arch in each group was measured as follows: cholesterol diet group, 0.22 ± 0.04; cholesterol diet plus probucol group, 0.10 ± 0.03; cholesterol diet plus Sal B group, 0.22 ± 0.04. Among these groups, the ratio of intimal thickness in the aortic arch in the cholesterol diet plus probucol and cholesterol diet plus Sal B groups was significantly lower than that in the cholesterol diet group ($P < 0.05$), and there was no difference between the cholesterol diet plus probucol and cholesterol diet plus Sal B groups (Figs 6A–6D).

The ratio of intimal thickness in the thoracic aorta in each group was measured as follows: cholesterol diet group, 0.10 ± 0.02; cholesterol diet plus probucol group, 0.03 ± 0.01; cholesterol diet plus Sal B group, 0.02 ± 0.01. Among these groups, the ratio of intimal thickness in the thoracic aorta in the cholesterol diet plus probucol and cholesterol diet plus Sal B groups was significantly

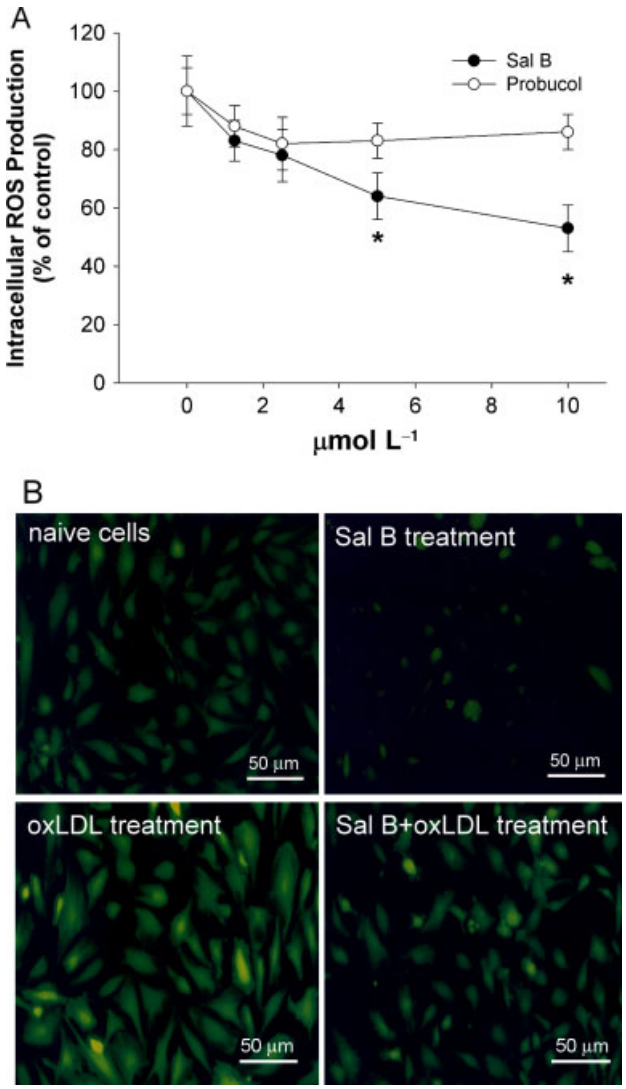

Figure 3. Effect of Sal B and probucol on intracellular ROS production. (A) HAECs were treated with various concentrations of probucol or Sal B followed by 30 μg mL^{-1} oxLDL for 1 h. ROS were measured using DCFH-DA. ROS levels were significantly decreased in HAECs treated with 5 or 10 μmol L^{-1} Sal B. *$P < 0.05$ compared with oxLDL group. (B) Flurorescent images of representative control, Sal B-treated, oxLDL-treated and (Sal B + oxLDL)-treated HAECs.

Table 1. Body weight and plasma levels of blood urea nitrogen (BUN), creatinine, glutamate oxaloacetate transaminase (GOT), glutamate pyruvtate transaminase (GPT), triglyceride and cholesterol at 0 (baseline), 2, 4 and 6 weeks in different groups of rabbits

Parameter	Week	Control ($n = 10$)	Probucol ($n = 10$)	Sal B ($n = 10$)
Body weight (kg)	0	2.0 ± 0.1	2.1 ± 0.1	2.0 ± 0.1
	2	2.1 ± 0.1	2.2 ± 0.1	2.1 ± 0.1
	4	2.1 ± 0.1	2.1 ± 0.1	2.1 ± 0.1
	6	2.1 ± 0.1	2.2 ± 0.1	2.1 ± 0.1
BUN (mg dL^{-1})	0	19.8 ± 1.7	18.5 ± 2.1	15.1 ± 1.7
	2	14.0 ± 2.9	12.3 ± 2.3	16.5 ± 3.0
	4	14.9 ± 1.4	19.2 ± 2.6	17.2 ± 1.2
	6	21.0 ± 1.9	23.1 ± 5.0	23.0 ± 3.7
Creatinine (mg dL^{-1})	0	1.1 ± 0.2	1.2 ± 0.2	1.2 ± 0.1
	2	1.3 ± 0.2	1.3 ± 0.2	1.4 ± 0.1
	4	1.2 ± 0.1	1.4 ± 0.1	1.3 ± 0.1
	6	1.1 ± 0.2	1.3 ± 0.2	1.6 ± 0.1
GOT (U L^{-1})	0	10.3 ± 5.2	8.8 ± 4.1	16.5 ± 5.0
	2	11.8 ± 4.1	23.7 ± 7.9	13.1 ± 2.8
	4	23.6 ± 6.5	24.1 ± 4.9	10.6 ± 2.1
	6	46.1 ± 8.7*	29.3 ± 6.5*	20.4 ± 2.9†
GPT (U L^{-1})	0	29.0 ± 6.7	26.5 ± 8.7	29.9 ± 8.0
	2	55.9 ± 8.1*	59.5 ± 8.2*	68.3 ± 11.0*
	4	79.6 ± 13.5*	56.3 ± 7.0*	72.2 ± 6.9*
	6	79.2 ± 13.5*	54.8 ± 10.6*	47.1 ± 5.2†
Triglyceride (mg dL^{-1})	0	51.1 ± 9.1	50.7 ± 7.3	36.3 ± 5.6
	2	59.1 ± 13.3	68.5 ± 13.5	59.5 ± 14.6
	4	70.6 ± 15.8	89.9 ± 18.3	69.5 ± 10.4
	6	66.5 ± 9.1	82.5 ± 16.0	52.4 ± 6.8
Cholesterol (mg dL^{-1})	0	68.9 ± 8.4	101.4 ± 14.3	86.3 ± 14.6
	2	1179.0 ± 184.6*	1208.0 ± 174.9*	1157.1 ± 153.4*
	4	1713.5 ± 191.7*	1522.0 ± 192.5*	1792.2 ± 119.6*
	6	2316.0 ± 324.4*	1638.0 ± 207.7*	2100.0 ± 95.5*

Data are expressed as mean ± SEM. * $P < 0.05$ compared with week 0 group; † $P < 0.05$ compared with control group.

lower than that in the cholesterol diet group ($P < 0.05$), and there was no difference between the cholesterol diet plus probucol and cholesterol diet plus Sal B groups (Figs 6E–6H).

Sal B reduction of neointimal formation in abdominal aorta after balloon injury of cholesterol-fed rabbits

The abdominal aorta of animals in each group was subjected to balloon injury. The neointima in the abdominal aorta in the cholesterol diet group was thicker and showed vacuoles when observed by microscopy. The neointima in the abdominal aorta in the cholesterol diet plus probucol and cholesterol diet plus Sal B groups was thinner than that in the cholesterol diet group and showed fewer vacuoles when observed by microscopy.

The ratio of intimal thickness in the abdominal aorta in each group was measured as follows: cholesterol diet group, 0.58 ± 0.03; cholesterol diet plus probucol group, 0.33 ± 0.03; cholesterol diet plus Sal B group, 0.26 ± 0.03. The ratio of neointima in the abdominal aorta in the cholesterol diet plus probucol and cholesterol diet plus Sal B groups was significantly lower than that in the cholesterol diet group ($P < 0.05$) (Figs 6I–6L).

DISCUSSION

In this study we have demonstrated that Sal B (i) decreases Cu^{2+}- and endothelial cell-induced LDL oxidation, (ii) reduces oxLDL-induced ROS production and cytotoxicity in endothelial cells and (iii) inhibits serum LDL oxidation, reduces atherosclerotic lesions and diminishes neointimal formation induced by high cholesterol in an animal model.

Hyperlipidaemia imparts a high risk of atherogenesis. It increases the permeability of vascular endothelial cells and the attachment of monocytes and endothelium, accelerates the movement of LDL through the subendothelial space and stimulates vascular smooth muscle cell migration, which can result in atherosclerosis.[1] Previous

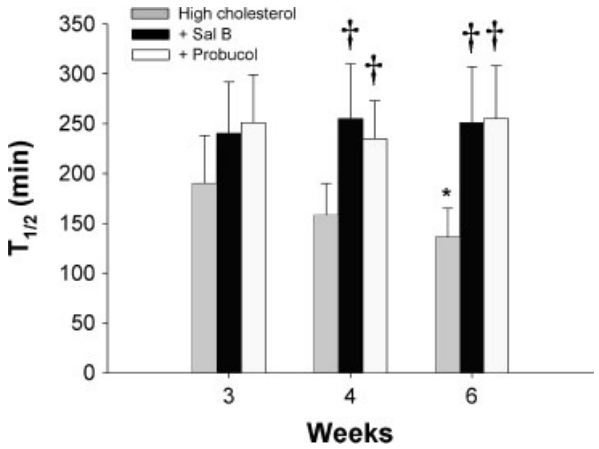

Figure 4. Effect of Sal B and probucol on inhibition of Cu^{2+}-induced oxidation of LDL from high-cholesterol-fed rabbits. Rabbits were divided into three groups and fed one of the following diets: high-cholesterol diet; Sal B + high-cholesterol diet; probucol + high-cholesterol diet. After 3, 4 and 6 weeks, LDL oxidation induced by $CuSO_4$ was measured as described in the text. Sal B and probucol prolonged the reaction time ($T_{1/2}$) of LDL oxidation at 4 and 6 weeks. $^*P < 0.05$ compared with high-cholesterol diet for 3 weeks; $^\dagger P < 0.05$ compared with high-cholesterol diet for indicated week.

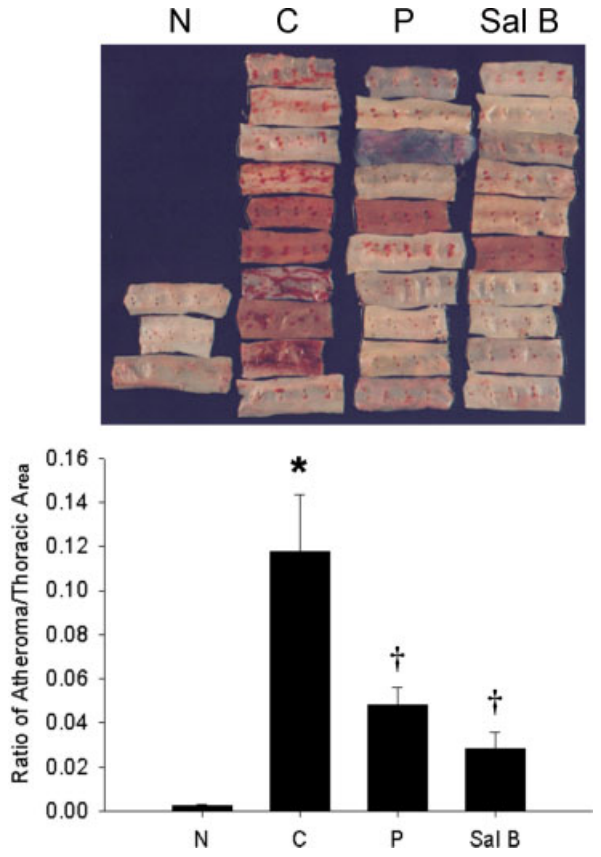

Figure 5. Rabbits were fed one of the following diets: N, normal diet; C, high-cholesterol diet; P, probucol plus high-cholesterol diet; Sal B, Sal B plus high-cholesterol diet. After feeding for 6 weeks, the atheroma area in each thoracic aorta was measured. (A) Sudan IV staining of thoracic aorta, with atherosclerotic areas stained in red. (B) By software analysis (atherosclerotic area/thoracic aorta area) it was found that areas of atherosclerosis decreased significantly in both the Sal B and probucol treatment groups. $^*P < 0.05$ compared with normal diet (N); $^\dagger P < 0.05$ compared with high-cholesterol diet (C).

studies have indicated that oxidation of LDL is a major event in cardiovascular diseases and that inhibition of LDL oxidation by antioxidants reduces the development of atherosclerosis.[32] For example, vitamin E prevents LDL oxidation induced by homocysteine,[33] while green tea prevents LDL oxidation induced by endothelial cells.[30] The water-soluble extract of *S. miltiorrhiza*, which is composed of protocatechuic acid, protocatechuic aldehyde, caffeic acid, rosmarinic acid, lithospermic acid, Sal A, B and C, 3,4-dihydroxybenzoic acid and 3,4-dihydroxyphenyl lactic acid, has potential antioxidant properties.[34] The water-soluble extract of *S. miltiorrhiza* suppresses the oxidation of human LDL in aortic smooth muscle cells.[35]

In a previous study we found that the water-soluble extract of *S. miltiorrhiza* not only reduces cholesterol levels in animal blood but also reduces LDL oxidation.[10] Sal A eliminates ROS released by neutrophils and diminishes acute injury in the rat liver induced by CCl_4.[36,37] The role of Sal B as an antioxidant was unclear, so we studied the function of Sal B in oxLDL-treated endothelial cells and cholesterol-fed endothelium-denuded rabbits. We found that Sal B did not modulate the plasma level of cholesterol but reduced GOT and GPT levels. This finding corresponds with a previous report that Sal B may protect liver function.[38]

In animals, oxygen is metabolised and transformed into ROS, which may oxidise LDL through the enzyme and electron carrier system; a vicious cycle is thus formed and oxLDL may be a stimulator of ROS.[39,40] Therefore endothelial cells were pretreated with Sal B and ROS production was analysed. Our results show that Sal B reduces ROS production in oxLDL-treated cells. Previous reports indicate that the water-soluble extract of *S. miltiorrhiza* protects the liver and red blood cells from injury by reducing oxidative stress[38] and reperfusion injury.[34] Our studies show that Sal B inhibits LDL oxidation *in vitro* and in an animal model. Sal B may have a role as a free radical scavenger and may prevent cardiovascular diseases.

The functions of the liposoluble components of *S. miltiorrhiza* have been studied previously.[41] The hydrophilic components of *S. miltiorrhiza* are thought to have antioxidant properties. Some studies indicate that the hydrophilic components of *S. miltiorrhiza* may increase the level of LDL-containing vitamin E and inhibit atherosclerosis in rabbits fed a high-cholesterol diet.[10] In the present study we demonstrated that Sal B treatment decreased neointimal hyperplasia of the abdominal aorta in endothelium-denuded cholesterol-fed rabbits. In the process of atherosclerosis formation it is essential that macrophages uptake a substantial amount of LDL. OxLDL induces the formation of foam cells by being taken into the cells through scavenger receptors on the surface of macrophages. Macrophage scavenger receptors (MSRs) have a high affinity for oxLDL. Many anionic ligands are recognised by MSRs, including lipopolysaccharides, polyribonucleotides and lipoteicholic acids; these ligands induce signalling in the macrophage.[42] The chemical structure of Sal B is regarded as anionic group-rich after hydrolysis *in vivo*. This anionic group-rich structure would bind to MSRs to block signalling in the macrophage and further inhibit LDL oxidation. Whether Sal B can enhance antioxidation systems such as superoxide dismutase *in vivo* is not known.

The present study demonstrated that Sal B has protective effects against hyperlipidaemia-induced atherosclerosis. This protective effect is most likely mediated by the inhibitory effect of Sal B on LDL oxidation. The protective activity of Sal B against LDL

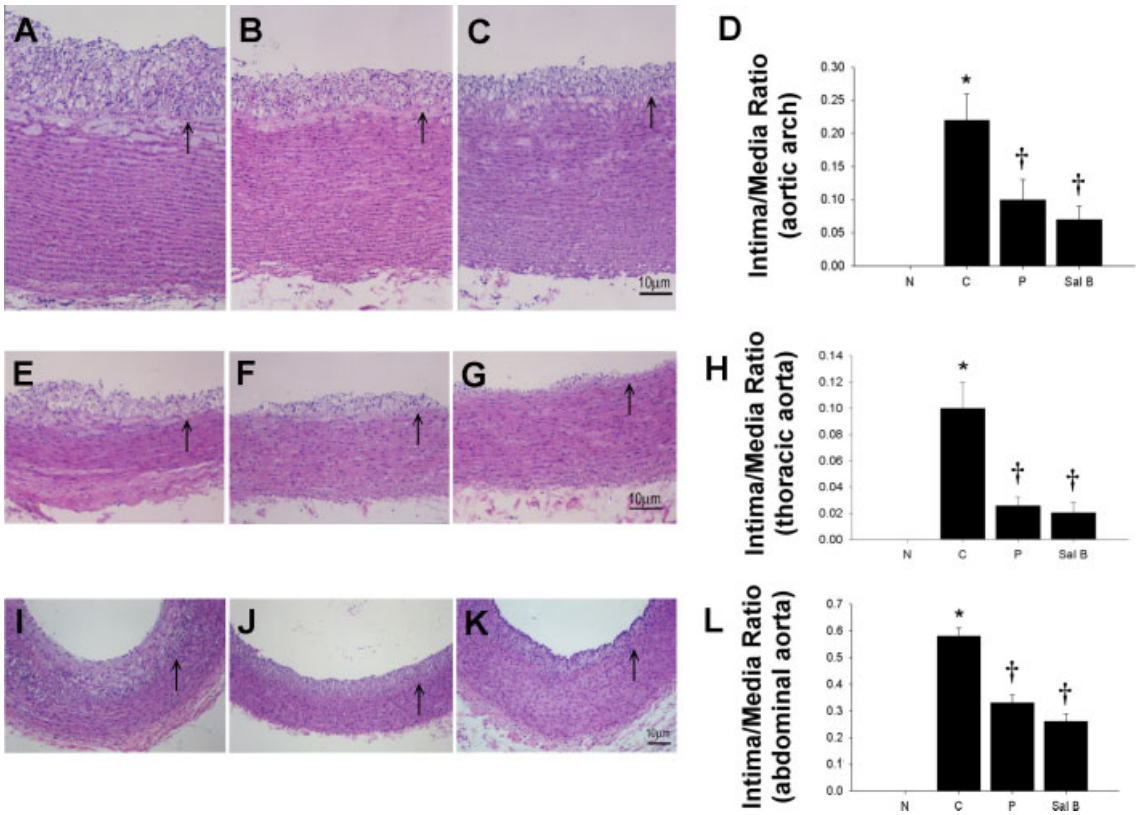

Figure 6. Rabbits were fed one of the following diets: N, normal diet; C, high-cholesterol diet; P, probucol plus high-cholesterol diet; Sal B, Sal B plus high-cholesterol diet. (A–D) Aortic arch sections were stained with H&E. The intimal area decreased significantly in the Sal B and probucol treatment groups. (E–H) Thoracic aorta sections were stained with H&E. The intimal area decreased significantly in the Sal B and probucol treatment groups. (I–L) abdominal aorta sections were stained with H&E. Restenosis was reduced significantly in the Sal B and probucol treatment groups. Arrows indicate the internal elastic laminae. $^{*}P < 0.05$ compared with normal diet (N); $^{\dagger}P < 0.05$ compared with high-cholesterol diet (C).

oxidation appears to be related to the antioxidant activity of Sal B. Our study underscores the potential of Sal B in the treatment of cardiovascular diseases.

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