S11 - 3

Novel Antioxidants and Atherosclerosis

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ABSTRACT

Coronary heart disease (CHD) has been the number one killer in western society for a long time, and CHD in most instances is due to atherosclerosis. One of the earliest events in atherogenesis is the intracellular accumulation of lipids, particularly cholesterol esters, in the aortic intima. The lipids presumably came from the uptake of plasma lipoproteins, particularly from LDL. These foam cells were identified as being predominantly as macrophages. Currently, it is believed that oxidation of low density lipoprotein (LDL) might contribute to the generation of foam cells. An outcome of the oxidation hypothesis is that the consumption of antioxidants would be beneficial. In this study, Boldine, an alkaloid of *Peumus boldus* was tested for their antioxidant potency both in, *in vitro* oxidation system and in mouse models. Boldine decreased the *ex-vivo* oxidation of Low-density lipoprotein (LDL). *In vivo* studies were performed to study the effect of these compounds on the atherosclerotic lesion formation in LDL r-/- mice. Three groups of LDL r-/- mice (N=12 each) were fed an atherogenic diet. Group 1 was given vehicle and group 2 and 3 were given 1 and 5 mg of Boldine/day in addition to the atherogenic diet. The results indicated that there was a decrease in lesion formation reaching a 40% reduction due to Boldine compared to controls. The *in vivo* tolerance of Boldine in humans (has been used as an herbal medicine in other diseases) should make it an attractive alternative to vitamin E.

Key words: low density lipoprotein, lipid peroxidation, Boldo, LDL receptor knock out mice, atherosclerotic lesions

INTRODUCTION

Atherosclerosis involves three processes, oxidation, inflammation and hypercholesterolemia (1). This led to a plethora of studies to test the use of antioxidants, anti-inflammatory agents and cholesterol-lowering agents to reduce atherosclerosis in both human and animal models. Antioxidants such as vitamin E and beta-carotene were successful in animal studies but were of little consequence in human studies (2). Other antioxidants such as butylated hydroxy toluene and diphenyl-phenylene diamine proved clearly cytotoxic (2) but were effective in preventing atherosclerosis in experimental animals. Despite the availability of a vast number of antioxidants, very few have been tested *in vivo* (3). Several cholesterol-lowering drugs were successful in decreasing atherosclerosis in animal models and in humans (4). However, there is still a controversy if statins alone are enough to reduce atherosclerosis or antioxidants should be included along with a statin therapy (5).

In search for a better antioxidant, in the present study the effects of a naturally occurring plant alkaloid Boldine (Fig. 1) on the atherosclerotic lesion formation in LDL receptor knockout mice were studied. Boldine, an antioxidant alkaloid isolated from the leaf and bark of Boldo (*Peumus boldus)* has been well established to have free radical scavenger and hepatoprotective properties (6).

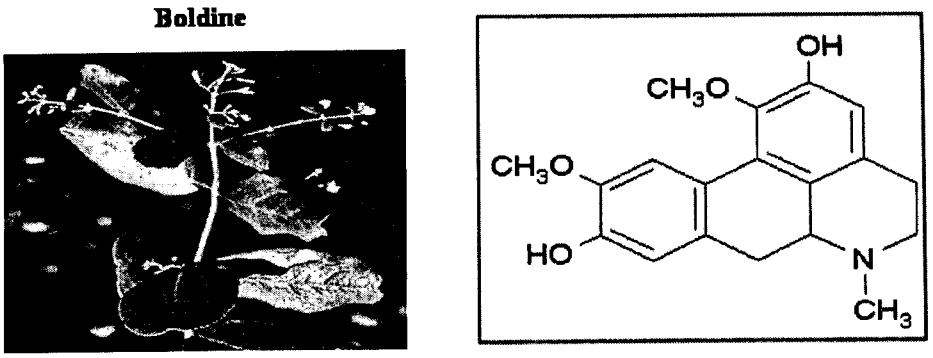

Fig. 1. Chemical structures of Boldine.

In the present study we used LDL receptor-deficient (LDL r-/-) male mice, to study the effects of boldine on the atherosclerotic lesion formation. The mice were fed antioxidants along with a high-cholesterol diet for 12 weeks and the atherosclerotic lesion formation along with the lipid levels and oxidative stress markers were measured.

MATERIALS AND METHODS

LDL receptor knockout mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The Emory Animal Care Committee approved all protocols and animals were treated in compliance with Emory University Animal Committee regulations. The high fat diet (TD88137) was purchased from Harland Teklad (Madison, WI). The TD88137 had 210 g/kg fat and 1.5 g/kg cholesterol. Boldine, secondary antibodies, protease inhibitors, alkaline-phosphatase substrates etc, were all purchased from Sigma Chemical Co. (St. Louis, MO). All blood-drawing supplies were obtained from Fisher Scientific.

Isolation of LDL

LDL was isolated from heparinized plasma from normal human volunteers using a Beckman TL-100 tabletop ultracentrifuge. Single spin gradient isolation was done adjusting 2 mL of plasma to a d=1.31 with potassium bromide (KBr) layered with saline and spun at 100,000 rpm for 1 hour. The isolated samples were respun at d=1.21 to concentrate and purify the LDL from any albumin contamination. The isolation was carried out without any EDTA and was completed in less than 3 hours. The isolated LDL was dialyzed against phosphate buffered saline (PBS) at 4°C for 4～6 hours (7). The purity of the isolated LDL samples was established by agarose and acrylamide gel electrophoresis (8).

Protein determination

Protein was determined by the method of Lowry *et al.* (9) using bovine serum albumin as the standard.

In vitro oxidation of LDL by Boldine

LDL samples were subjected to oxidation immediately after isolation. Typically, 100 μg/mL of LDL was incubated in PBS with 5 μM copper or 1U HRP with 50 μM H_2O_2 in the presence or absence of Boldine (0.5～5 μM ethanolic solution). The oxidation of LDL was followed continuously by measuring the formation of conjugated dienes at OD 234 nm in an SLM-Aminco DB-3500 spectrophotometer equipped with a 12-chamber cuvette changer. Samples and references were measured continuously for periods of up to 6～8 hours.

Study design

LDL receptor knockout male mice (n=36) 3～4 weeks of age were randomly divided into three groups with

12 mice in each group. Blood for baseline data were collected by retro-orbital bleeding after overnight food deprivation. All three groups were fed high-fat diet (TD88137: 210 g/kg fat and 1.5 g/kg cholesterol). Group one was fed 10 μL of ethanol (vehicle), group 2 was fed 1 mg (10 μL of ethanolic solution) of Boldine and group 3 was fed 5 mg (20 μL of ethanolic solution) of Boldine per mouse per day, five times a week for 12 weeks. Weekly food intakes and body weights were measured.

Collection of plasma, preparation of arterial samples and quantification of aortic lesion areas

After 12 weeks of treatment mice from both studies were sacrificed by CO_2 asphyxia and fasting blood was drawn in heparinized tubes from the inferior vena cava or by heart puncture. Red blood cells and plasma were immediately separated by centrifugation (2,000×g, 10 min at 4°C) and then frozen at -80°C. The aortic trunk was washed with cold PBS containing 10 mg/L aprotinin and 0.1 mmol/L PMSF through the left ventricle. The dissection of the aorta was performed under a stereomicroscope from the iliac bifurcation up to the heart, including the beginning of the carotid and subclavian arteries. The excess fat and connective tissue was carefully removed and the aorta was opened longitudinally and pinned up on black wax for *en face* observation (10,11). In order to use the aortas for future Western blot and enzyme assay analysis, no staining was used to visualize the lesions. After capturing of different areas of the aorta, the lesions were circled on a printout under direct microscopic observation. Lesion areas were quantified using Adobe Photoshop®. Pixels were transformed to mm^2 by using a microscopic standard scale treated under the same condition as the aortas.

Cholesterol profile measurements

Fast Phase Liquid Chromatography (FPLC) was used to measure plasma total cholesterol (TC), LDL, high-density lipoprotein (HDL) and triglyceride (TG) levels as described by Innis-Whitehouse (12). Whole plasma (300 μL) was used to determine plasma cholesterol levels.

Statistical analysis

Data is expressed as sample mean±standard deviations. Outliers were defined as data points greater than ±2 standard deviations from the mean. ANOVA was used as the test for statistical significance. Differences with $p< 0.05$ were accepted as significant. When significant effects were found, post hoc comparisons of means was done using the Bonferroni test. SPSS program was used for statistical analysis.

RESULTS

Inhibition of *in vitro* oxidation of LDL by Boldine

Boldine inhibited copper and peroxidase mediated oxidation of LDL in a concentration dependent manner. Increasing concentrations of Boldine were incubated with 100 μg/mL of LDL in the presence of 5 M of copper or peroxidase (HRP 1U/50 μM H_2O_2). Fig. 2a and b shows the effect of the addition of Boldine at concentrations (0.5 to 2.5 μM) on the formation of conjugated diene during the oxidation of LDL. As seen in the figures there was a concentration dependent increase in the lag time, suggesting an apparent antioxidant effect.

Protection of atherosclerosis by Boldine in LDL r-/- mice

LDL receptor knockout mice on atherosclerotic diet were given 1mg and 5 mg of Boldine for 12 weeks. The mice were euthanized after 12 weeks, the time necessary to observe and quantify atherosclerotic lesions by *en face* preparation. Fig. 3 shows a representative *en face* aortic preparation for the three groups of mice. As seen the lesions were mainly localized in the aortic arch, even though some lesion were found in the other areas. The

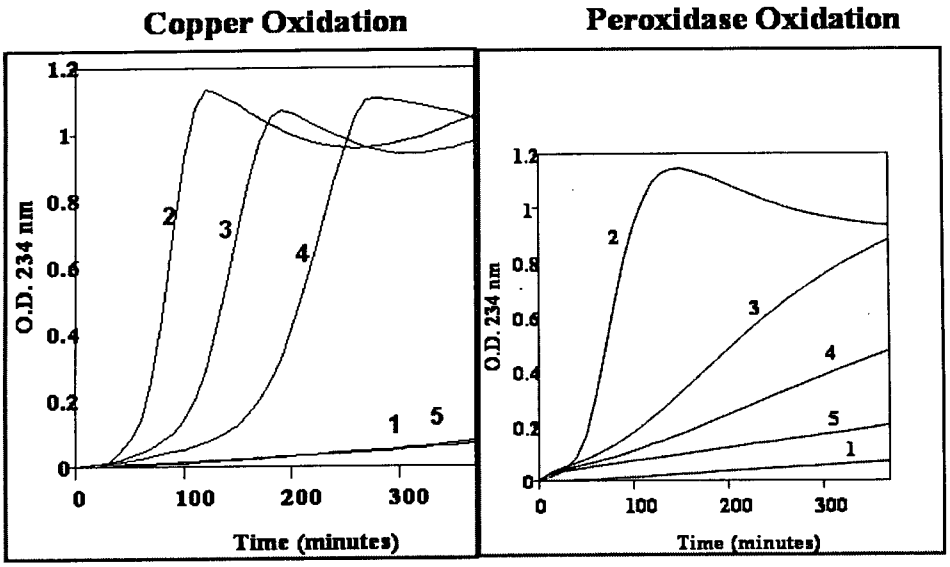

Fig. 2. Effect of Boldine on the oxidation of LDL by copper and peroxidase. LDL was incubated with 5 μM copper or 1U HRP with 50 μM H_2O_2 in the presence or absence of specified concentrations of Boldine. OD at 234 nm was monitored continuously for 300 minutes. The figure represents results from a typical experiment from over six individual experiments. 1, Control LDL without copper or HRP/H_2O_2; 2, LDL incubated with 5 μM copper or 1U HRP with 50 μM H_2O_2; 3, 5 μM copper or 1U HRP with 50 μM H_2O_2 and 0.5 μM Boldine; 4, 5 μM copper or 1U HRP with 50 μM H_2O_2 and 1 M Boldine; 5, 5 μM copper or 1U HRP with 50 μM H_2O_2 and 2.5 μM Boldine.

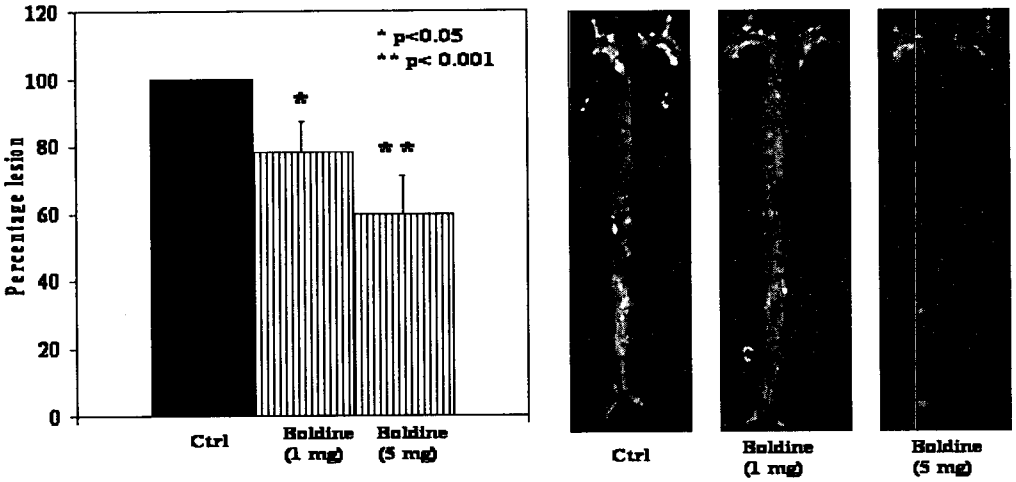

Fig. 3. Aortic lesion areas and representative samples of aortas from LDL r-/- mice. Computerized quantification of lesion area was performed on aortas of LDL r-/- mice (n=12 each) fed atherogenic diet for 12 weeks with or without 1 and 5 mg of Boldine. *Right Panel*: Pictures of representative aortas from each group, illustrating the size of lesion formation. *Left Panel*: Decrease in lesion area by 22% and 40% by 1 and 5 mg/day of boldine compared to atherogenic diet fed control animals. Lesion areas results are expressed as % control group. ANOVA was used as the test for statistical significance. Differences with $p<0.05$ were accepted as significant. When significant effects were found, post hoc comparisons of means was done using the Bonferroni test. SPSS program was used for statistical analysis.

areas of lesions were significantly decreased in the mice fed Boldine. Fig. 3 (left panel), represents the percentage representation of the aortic lesions from all mice from each group. As seen there was a significant decrease in the lesion area by 1 mg of Boldine 22% (ANOVA, $p=0.05$) and 40% lesser lesions in the group that was fed 5

mg/day boldine compared to the controls (ANOVA, p=0.001). This experiment suggests that Boldine decreases lesion size in a dose dependent fashion.

DISCUSSION

Boldine is a natural compound with a well-established free radical scavenger and hepatoprotective properties (13,14). Several earlier studies have suggested Boldine to have antioxidant activity due to its ability to decrease oxidant induced lipid peroxidation *in vitro* and in streptozotocin induced diabetic rabbits (15). Ours is the first study to show its ability to decrease the *ex vivo* oxidizability of LDL by both copper and peroxidase system.

Boldine at both concentrations decreased the atherosclerotic lesion in a significant manner. There was a slight decrease in the total and LDL cholesterol and a slight elevation in the triglyceride levels (*data not shown*) by Boldine even though not significant. This observation suggests that boldine protected LDL r-/- mice from atherosclerosis and this protection was independent of changes in plasma cholesterol levels. The effects may therefore be attributed to its antioxidant properties. This was confirmed by the decrease in the autoantibodies to lipid peroxide modified protein formation *in vivo* (*data not shown*) and the whole plasma oxidation by copper. However, no significant differences in 8-isoprostane levels were observed in these three groups (*data not shown*). We were not surprised with these results because there have been studies in the literature that show that antioxidant feeding does not alter F2 isoprostane levels (16).

The study showed that Boldine had a dose dependent effect on all the above observations such as the decrease in atherosclerotic lesion formation, slight decrease in total and LDL cholesterol and slight increase in triglyceride, decrease in autoantibodies and decrease in whole plasma oxidation. Several studies are being conducted to understand the actual therapeutic potential of Boldine as an antioxidant. The antioxidative capacity of Boldine has been attributed to the presence of the biphenyl group in its structure (17). Studies by Jimenez and Speisky (13) have studied the pharmacokinetics of this compound showing that it is rapidly absorbed and preferentially concentrates in the liver. The SGOT levels measured in these animals suggested that boldine at the doses tested were non-toxic.

The choice of a good antioxidant to decrease atherosclerosis in both animal and human trials is still controversial. However, the compound used in this study, Boldine may be a better choice in human clinical trials due to its already available pharmacokinetics and its use in the treatment of gastrointestinal disorders (6,18).

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