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Inhibition effects of flavonoid on Oxidation of Human Low Density Lipoprotein

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Abstract.

This study was designed to investigate the antioxidative activity of a substance isolated from. The antioxidative activity of procyanidine was higher than that of dℓ-tocopherol and BHA on low-density lipoprotein (LDL) oxidation by thiobarbituric acid reactive substance (TBARS). Procyanidine inhibited the copper-mediated oxidation of human LDL in a dose dependent manner with almost complete inhibition at 60μg/mL. Procyanidine at a concentration of 80μg/mL in hibited oxidation of LDL induced by J774. LDL oxidized by copper-mediated or cell-induced oxidation was degraded at a much greater rate than native LDL. These results suggested the importance of further research to procyanidine in the investigation of atherosclerosis and free radical-induced injury.

Introduction

Oxidative mcolification of low density lipoprotein (LDL) that alter physicochemical and biological properties of the particles, are thought play a central role in atherogenesis (1). An early event in atherogenesis is the accumulation of lipid-laden foam cells in the arterial intima, which can progress to fatty streaks and plaques. Most of foam cells are likely derived from resident tissue macrophages, which can lead to cellular cholesterol accumulation(2). Oxidized LDL that has entered the artery wall and then accumulated in foams cells would affect atherosclerotic progress(3). LDL oxidation was used to test the effectiveness of antioxidants to slow atherosclerosis in animal models(4). It was observed that antioxidants or drugs with antioxidant activity were consistently able to reduce the extent of atherosclerosis. The exact mechanism by which LDL undergoes oxidatioin *in vivo* is not yet fully clear but there is little doubt that it involves free radical peroxidation of LDL. Since oxidized LDL seems to play a role in the development

of atherosclerosis, prevention by antioxidants such as probucol. β-carotene, vitamin E, vitamin C, and flavonoids may be a therapeutic option.

The recent discovery of natural antioxidants may lead to replacement of the synthetic antioxidants which are widely used at present. Antioxidants from natural substances such as edible plants, species, and herbs have been widely investstigated. A number of naturally occurring antioxidant compounds have been found to strengthen the resistance of LDL to oxidative modification *in vitro* and *in vivo*(5). Attention has been focused recently on the importance of the protective defense systems in living cells against damage caused by LDL.

Procyanidine, polyphenol oligomers arising from condensation of monomeric units of flavan-3-ol and flavan-3,4 diol, widely diffused in the vegetable kingdom, have been shown to have positive effects on the biochemical propertice. The present studies were carried out to the ascertain antioxidative effect of procyanidine on LDL oxidation.

Materials and methods

Material Procyanidine was obtained from Dr. Greenspan. (College of Pharmacy, The University of Georgia, USA).

Lipoproteins Human LDL was isolated from the blood of healthy man by ultracentrifugation and dialyzed extensively against 0.9% (w/v) NaCl, 0.004% (w/v) EDTA, pH 7.4. Prior to oxidation, LDL was dialyzed against phosphate-buffered saline, pH 7.4, to remove the EDTA.

Cell culture Transformed mouse macrophage J774 cells were maintained in Ham's F-10 supplemented with 10% (v/v) fetal calf serum, $NaHC0_3$ (2 g/liter) and 4 mM Hepes. pH 8.1 . A series of antibiotics was included in rotation in the medium. The cells were cultured routinely in large dishes (90 mm diameter) in 10 mL of medium and plated out into smaller dishes (60 mm diameter) containing 2 mL of medium. Cultures were maintained in a humidified incubator at 37℃ and the medium was changed every 48 h.

Oxidation of LDL Two different methods were used to examine the effect of the novel com-pound on the oxidation of LDL. In the first method, LDL (100mg Protein/mL) was incubated in the presence of 5 μM $CuS0_4$ in phosphatebuffered saline, pH 7.4, at 37℃ for 18 h. In the second method, LDL (100 μg protein/mL) was incubated with J774 macrophages in Ham's F-10 culture medium for 24 h at 37℃. To examine the effect of antioxidant on LDL, the reaction was stopped by the

addition of EDTA (final concentration of 10 μM) and placing the lipoproteins on ice.

Assay of thiobarbituric Acid-Reactive Substances (TBARS) TBARS levels were determined spectrophotometrically. To 0.1mL aliquots of post incubation mixture and tetramethoxypropane standards were added 1 mL of 20% trichloroacetic acid and 1 mL of 1% thithio barbituric acid containing EDTA. Tubes were placed in a boiling water bath for 30 min. After cooling, tubes were centrifuged at 1500g for 15 min. Absorbance of the supernatant was measured at 532 nm.

Detection of conjugated dienes The formation of conjugated dienes associated with oxidized LDL was measured by monitoring the absorption at 234 nm using a UV-VIS spectrophotometer. Briefly, 1 mL of an LDL solution (100 mg LDL, protein/mL) in phosphate-buffered saline, pH 7.4, was incubated with 5 μM $CuSO_4$ at 37℃ in the presence or absence of test agents, and then the absorbance at 234 am was measured every 30 min. The formation of conjugated dienes in control solutions containing antioxidant in the absence of LDL and 5 μ M $CuSO_4$ was also determined.

Results and Discussion

Antioxidative effect on human LDL The antioxidative effect of procyanidine on the oxidation of LDL, as measured by production of TBARS, was initially examined at various concentrations of procyanidine, procyanidine demonstrated a concentration-dependent inhibition of the production of oxidized LDL by Cu^{2+}-mediated LDL oxidation (Fig. 1).

Procyanidin showed a dose-dependent inhibition of Cu^{2+}-mediated LDL oxidation after 6 and 24 h of incubation. At a concentration of 60 μg/mL procyanidine, the oxidation of LDL was approximately 30% of that observed in the absence of procyanidine. Although research supports the *in vivo* existence of oxidized LDL, the most persuasive data on the role of oxidized LDL in atherogenesis derives from studies showing that antioxidants prevent atherosclerosis in animal models while some antioxidants such as BHA and BHT prevented atherosclerosis in animals, but their side effects preclude their use in human subjects. Oxidation of LDL in the presence of copper was maximal between 2 and 3 h of incubation; oxidation for 24 h of incubation was almost four fold greater than at 4 h. This may reflect the level of endogenous antioxidants present in the LDL preparation, which may vary with individual donors. For example, vitamin E as dietary

antioxidant con- tained to partition into the LDL fraction which protection against

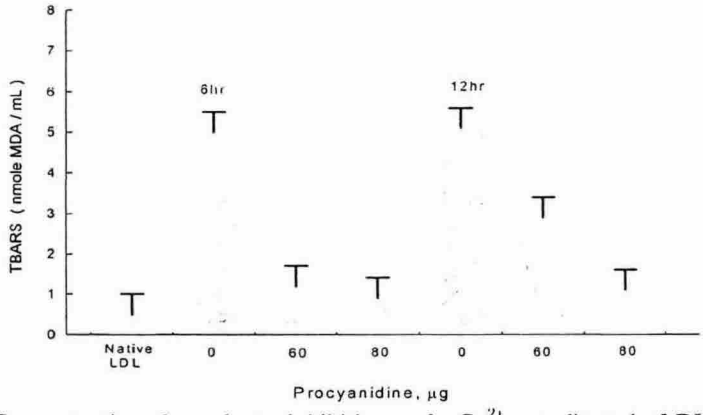

Fig. 1. Concentration-dependent inhibition of Cu^{2+} medicated LDL oxidation by procyandine. LDL (100μg protein/mL) was incubated for 6 and 24 h at 37℃ in phosphate-buffered saline containing 5μM $CuSO_4$ in the presence or absence of increasing concentration of procyanidine. The lipoperoxide content was measured as thiobarbituric acid-reactive substance (TBARS) and is expressed as nanomoles of malondialdehyde(MDA) equivalents per mililiter. Results are presented as means ±SEM of there to five independent experiments.

LDL oxidation, but LDL from donors who smoker occurred more susceptible to oxidation than non-smoker. Human LDL was oxidized by mouse J774 macrophages, in a time-dependent manner and the production of TBARS reached a plateau after 24 h of incubation. Therefore, all data relating to macrophages presented here were obtained following 24 h incubation. The production of TBARS in the presence or absence of the macrophages was 5.0 ± 0.1 nmol MDA/mL and 0.42±0.03 nmol MDA/mL protein, respectively. Increasing the number of macrophages per dish increased the production of TBARS (data not shown). The oxidation of LDL by J774 macrophages was completely inhibited in the presence of 80 μg/mL procyanidine in the culture medium. Although the mechanism of oxidation is not known, LDL oxidation may involve cellular lipoxyenases. The inhibition of macrophage induced oxidation by procyanidine is consistent with the role of lipoxygenase. Macrophages on endocytose and degrade oxidatively modified LDL via scavenger receptors at a much greater rate than native LDL and this property was used to assess, the protection afforded LDL by coincubation with procyanidine during the oxidation period. Many other cells

types have since been shown to oxidize LDL *in vitro*, e.g., mouse peritoneal macrophages. It was observed that protection by procyanidine on cell-induced LDL oxidation may be, in part, through its capacity to scavenge $0_2^{\cdot}$ radicals. However, inhibition of Cu^{2+}-mediated LDL oxidation by procyanidine was less marked than the oxidation by macrophages. The differences in procyanidine potency in the two systems might be due to the fact that oxidative modification is more complex than a simple free radical reaction. Moreover, the mechanism of LDL oxidation by transition metals still remains to be clarified.

Effects of Procyanidine on LDL oxidation and assay electrophoretic mobility Table 1 shows the effect of procyanidine on the electrophoretic mobility of LDL submitted to oxidative modification by macrophages. A marked increases from 1.67±0.11 to 1.03±0.01mm in the electrophoretic mobility of LDL incubated with macrophages for 24 h implies lipid peroxidation of LDL and an increase in negative charges on the LDL molecule. Procyanidine reduced the relative electrophoretic mobility of LDL in dose dependently. LDL oxidized by $CuSO_4$ displayed a greater electrophoretic mobility in agarose gels compared to native LDL. When LDL was incubated with 20 μM procyanidine, the electrophoretic mobility of oxidized LDL was only slightly greater than native LDL. Procyanidine inhibited the cell-induced oxidation of LDL as measured by lipoperoxide content of the electrophoretic mobility of LDL in agarose gels. Steinbrecher et at. demonstrated that LDL can be modified by the addition of fatty acid peroxidation in the absence of cells. This modified LDL possesses an enhanced electrophoretic mobility without the lipid constituents of LDL being oxidized.

Table 1. Effect of procyanidine oxidation as assessed by electropgoretic mobility.

Incubation conditions	Relative electrophoretic mobility	*P*
Native LDL	1.0	
LDL + cell + vehicle (control)	1.67±0.11	
LDL + cell + procyanidine 10μ*M*	1.54±0.02	
LDL + cell + procyanidine 20μ*M*	1.30±0.03	<0.05
LDL + cell + procyanidine 30μ*M*	1.12±0.01	<0.01
LDL + cell + procyanidine 40μ*M*	1.03±0.01	<0.014

"LDL (100 μg/mL) was incubated for 24 h in Hams F-10 medium in 35-min dishes containing macrophages in the presence or absence of procyanidine. The electrophoretic

mobility of LDL was determined in agarose gel as described in the text. Results are means ±SEM of three to five independent expeiments.

It is possible that oxidation of LDL mediated by macrophages in Ham's F-10 culture medium can also contribute to the modification of the LDL protein as determined by the enhanced electrophoretic mobility.

Effects of procyanidine on conjugated diene formation Fig. 2 shows the effects of procyanidine on the formation of conjugated dieses, a measurement of the LDL oxidative process. The conjugated dienes formed were significantly lower in the presence of procyanidine than the control. Incubation of LDL with Cu^{2+} produced a lag phase of 120 min before the onset of the propagation phase where polyunsaturated fatty acid underwent conversion to conjugated lipid hydroperoxides. However, in the presence of procyanidine at 60 or 80 μM, the lag phase and propagation phase were inhibitory. In agreement with the findings of Esterbauer et at. There was an initial lag period in the formation of conjugated dienes. Antioxidative activities of procyanidine were measured by 1.1-diphenyl-2- picrylhydrazyl (DPPH) and thiocynate methods and compared with BHA and α-tocopherol.

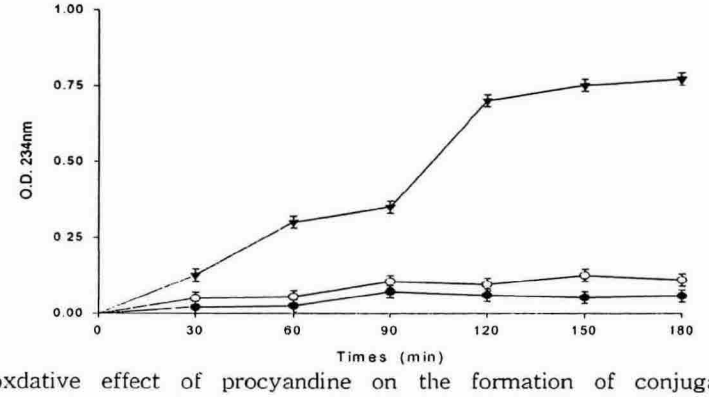

Fig. 2. Antioxdative effect of procyandine on the formation of conjugated dienes observed during the oxidation of LDL. (▼ : LDL +5 μg Cu^{2+} + 60μg Procyandine, ■ : LDL +5 μg Cu^{2+} + 80μg Procyandine, ● : LDL +5 μg Cu^{2+})

LDL (100μg protein/mL) was incubated by the addition of 5μM $CuSO_4$. The formation of conjugated dienes was a mesuare of LDL oxidation. Results are presented as means ±SEM of three to five independent experiments.

This study shows that procyanidine inhibited the Cu^{2+}-mediated oxidation of human LDL in a dose-dependent manner with complete inhibition at 60 μg/mL.

Procyanidine at a concentration of 80μg/mL also inhibited oxidation of LDL induced by the mouse transformed macrophage, J774. Oxidation of LDL increased macrophage uptake of LDL, which may contribute to the formation of Cu^{2+}-mediated or macrophage derived foam cells in early atherosclerotic lesions. From these reports, it appears that procyanidine is important in preventing the oxidative modification of LDL. Procyanidine may also play an important role in preventing the peroxidation of LDL *in vivo*, perhaps through regenerating lipid soluble antioxidants such as α-tocopherol. The antioxidant activity of procyanidine in LDL, plasma, or arterial walls may be important in preventing or reducing the progression of atherosclerosis by inhibiting the peroxidation of lipoproteins.

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