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Pine bark extract prevents low-density lipoprotein oxidation and regulates monocytic expression of antioxidant enzymes

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ABSTRACT

Polyphenols are widely distributed in leaves, seeds, bark, and flowers and considered to have beneficial effects on cardiovascular health. We hypothesized that the potent antioxidant properties of pine bark extract (PBE) are exerted by its ability to scavenge free radicals and induce antioxidant enzymes. Therefore, we investigated the effects of PBE on low-density lipoprotein (LDL) oxidation and the antioxidant defense system in monocytes. Oxidative susceptibility of LDL was determined by lag time assay in vitro and by using a human umbilical vein endothelial cell–mediated oxidation model. THP-1 monocytic cells were treated with PBE, and the expression of antioxidant enzymes was measured by real-time polymerase chain reaction and Western blot. Pine bark extract showed radical scavenging ability and significantly inhibited free radical-induced and endothelial cell-mediated LDL oxidation in vitro. Pine bark extract treatment resulted in increases in the expressions of antioxidant enzymes, glutathione peroxidase-1, catalase, and heme oxygenase-1 in THP-1 cells. In addition, PBE induced nuclear factor-erythroid-2–related factor 2 activation, which was accompanied by the activation of extracellular signal-regulated kinase and Akt despite a down-regulation of reactive oxygen species. After the monocyte investigations, we further examined the antioxidant effect after the intake of PBE by 10 healthy male volunteers. Pine bark extract significantly prolonged the lag time of LDL oxidation. Based on our findings, it appears that PBE enhances the antioxidant defense capacity of LDL and monocytes and may play a preventive role in atherosclerosis progression.

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Abbreviations: AMVN-CH_3O, 2,2-azobis-4-methoxy-2,4-dimethylvaleronitrile; ANOVA, analysis of variance; CAT, catalase; DCFH-DA, 2′,7′-dichlorofluorescein-diacetate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EDTA, ethylenediamine-tetraaceticacid; HO-1, heme oxygenase-1; HUVECs, human umbilical vein endothelial cells; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GPx, glutathione peroxidase; Keap-1, kelch-like ECH-associated protein 1; LDL, low-density lipoprotein; MDA, malondialdehyde; Nrf2, nuclear factor-erythroid-2–related factor 2; PBE, pine bark extract; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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1. Introduction

The oxidation of low-density lipoprotein (LDL), which triggers foam cell formation and local inflammation, is a well-known key step in the initiation of atherosclerosis [1–4]. The pivotal role of LDL oxidation in atherogenesis suggests that antioxidants may contribute to the prevention of cardiovascular diseases. Dietary antioxidants such as polyphenols are reported to prevent LDL oxidation, both in vitro and in vivo [5–9], and have protective effects against coronary heart disease, as evidenced by epidemiologic findings [10,11].

In addition to their direct radical scavenging effects, polyphenols are thought to be able to enhance endogenous antioxidative capacity. Oxidative stress results from an imbalance between antioxidants and reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide (H_2O_2), and hydroxyl radicals. The human body has the ability to protect itself against oxidative damage with exogenous antioxidants obtained through the diet as well as endogenous antioxidant enzymes (eg, superoxide dismutase [SOD], glutathione peroxidase [GPx], catalase, and heme oxygenase-1 [HO-1]). We, in addition to others, recently found that the level of LDL oxidation is associated with SOD activity in metabolic syndrome and in hypercholesterolemic patients [12,13]. The overexpression of catalase and HO-1 retarded atherosclerosis progression in apolipoprotein $E^{-/-}$ mice [14,15]. These cytoprotective enzymes share common transcriptional regulation through the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor-erythroid-2–related factor 2 (Nrf2) pathway [16]. A number of studies indicated an association between polyphenol and antioxidant enzyme expression via Nrf2 activation in vivo and in various cultured cell lines [17–19].

Monocytes are key cells in the progression of atherosclerosis because they play a regulatory role in the immune system and can differentiate into macrophages. Moreover, recent nutrigenomic studies suggested that peripheral blood mononuclear cells (PBMCs) are a promising target, as they can reflect the effects of dietary interventions at the level of gene expression [20].

Pine bark extract (PBE), which was obtained from pine trees that grow on the west coast of France (*Pinus maritima*), is used as a folk medicine for various diseases and is gaining popularity as a dietary supplement. In addition, its safety and tolerability for long-term human consumption was already established in a large study [21,22]. The main component of PBE is oligomeric proanthocyanidins [23], and studies have already revealed the antioxidative [24], anti-inflammatory [25], and antiplatelet effects [26] of PBE. In a clinical study, Devaraj et al [27] reported that supplementation of PBE (150 mg/d) for 6 weeks was followed by increased plasma antioxidant capacity in healthy subjects. Improvements in endothelial dysfunction and the oxidative index were also observed after supplementation with PBE (200 mg/d) for 8 weeks in coronary artery disease patients [28]. Although there are a limited number of clinical studies, the effects of PBE on the antioxidant defense capacity of LDL and monocytes have not been properly evaluated, especially in healthy subjects. We hypothesized that PBE could have potent antioxidant properties in scavenging free radicals and inducing antioxidant enzymes. To examine this possibility, we investigated the effects of PBE on LDL oxidation and monocytic antioxidant defense systems in vitro and in healthy human subjects.

2. Methods and materials

2.1. *Reagents*

PBE, Flavangenol, was provided by Toyo Shinyaku Co, Ltd (Saga, Japan). Total polyphenol content of PBE powder was greater than 70%, which consisted of 3.7% proanthocyanidin B1, 1.6% proanthocyanidin B3, 3.0% catechin, 0.23% epicatechin, and 40.7% unidentified trimer and tetramer-type oligomeric proanthocyanidin. The powder was dissolved in deionized water and used in the experiments. Micro BCA Protein Assay Kit was obtained from Pierce Laboratories (Rockford, IL, USA). M-PER Protein Extraction Reagent and NE-PER Nuclear and Cytoplasmic Extraction Reagents were purchased from Thermo Scientific (Rockford, IL, USA). RPMI 1640 medium, fetal bovine serum, and penicillin/streptomycin were acquired from GIBCO (Paisley, UK). Lonza Walkersville (Walkersville, MD, USA) supplied endothelial cell growth medium-2 and Ham F10. Ammonium chloride solution was purchased from Stem cell technologies (Vancouver, Canada). TaqMan Reverse Transcription Reagents and Power SYBR green polymerase chain reaction (PCR) mix were obtained from Applied Biosystems (Carlsbad, CA, USA). The Histopaque-1077 and 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) was obtained from Sigma-Aldrich (St Louis, MO, USA). All other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan), unless specified elsewhere.

2.2. *Experimental design*

To examine the antioxidant properties of PBE, we measured total polyphenol content by Folin-Ciocalteu assay (as described in Section 2.3) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (Section 2.4). Low-density lipoprotein oxidizability (in vitro) was determined by lag time assay using freshly prepared human LDL (2.6) and a human umbilical vein endothelial cell (HUVEC)–mediated oxidation model, which was followed by thiobarbituric acid reactive substance (TBARS) assay and LDL mobility assay (Section 2.8). The effect of PBE on monocytic expression of antioxidant enzyme was evaluated by real-time PCR (Section 2.9) and Western blot analysis (Section 2.10) in THP-1 cells. The upstream kinase activation and ROS production were also examined (Section 2.11). In addition, we conducted a human study to examine the effects of PBE intake on LDL oxidizability and PBMC's expression of antioxidant enzymes (Section 2.12).

2.3. *Polyphenolics analysis*

The total polyphenol content was determined by a Folin-Ciocalteu assay using (+)-catechin as the standard, based on a previous report [29]. In brief, Folin-Ciocalteu phenol reagent (Nakaraitesuku Co, Kyoto, Japan) was added to the sample and incubated in 1.5% $NaCO_3$ solution for 2 hours at 20°C, and the absorbance was then measured at 750 nm.

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