NUTRITION RESEARCH 35 (2015) 56–64

Available online at www.sciencedirect.com

ScienceDirect

www.nrjournal.com

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

Shiori Nakayama[a], *Yoshimi Kishimoto*[b], *Emi Saita*[a], *Norie Sugihara*[a], *Miku Toyozaki*[a], *Chie Taguchi*[b], *Mariko Tani*[a], *Tomoyasu Kamiya*[c], *Kazuo Kondo*[a,b,*]

[a] *Institute of Environmental Science for Human Life, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan*
[b] *Endowed Research Department "Food for Health," Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan*
[c] *Research and Development Division, Toyo Shinyaku Co Ltd, 7-28 Yayoigaoka, Tosu-shi, Saga 841-0005, Japan*

ARTICLE INFO

Article history:
Received 22 July 2014
Revised 29 September 2014
Accepted 15 October 2014

Keywords:
Pine bark extract
Polyphenol
Lipid peroxidation
Monocytes
Antioxidant
NF-E2–related factor 2

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.

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.

* Corresponding author. Tel.: +81 3 5978 5812; fax: +81 3 5978 2694.
E-mail address: kondo.kazuo@ocha.ac.jp (K. Kondo).

http://dx.doi.org/10.1016/j.nutres.2014.10.010
0271-5317/

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.

2.4. DPPH scavenging activity

We determined free radical–scavenging activity by using DPPH [29]. An aqueous solution of PBE powder was mixed with 1 mL of 0.2 mM DPPH in ethanol. After incubation for 20 minutes at 37°C, the absorbance of each solution was measured at 516 nm using a DU800 spectrophotometer (Beckman Coulter, Brea, CA, USA). The volume of PBE that was required to cause a 50% decrease in the absorbance at 516 nm relative to the solvent control was then calculated.

2.5. Preparation of LDL

Fasting normolipidemic adult volunteers provided the blood samples that were collected in sodium ethylenediaminetetraaceticacid (EDTA)–containing tubes. Plasma samples were immediately prepared by centrifugation, and LDL was separated by single-spin density gradient ultracentrifugation at 100 000 rpm for 40 minutes at 4°C. The LDL protein concentration was determined using a Micro BCA Protein Assay Kit. All volunteers provided informed consent prior to sample collection.

2.6. LDL lag time assay

To evaluate the antioxidant effect of the PBE on free radical–induced LDL lipid peroxidation, we measured LDL oxidizability, as previously described [30]. The prepared LDL samples (final concentration of protein: 70 μg/mL) were oxidized by 400 μM (for the in vitro study) or 200 μM (for the human study) of 2,2-azobis-4-methoxy-2,4-dimethylvaleronitrile (AMVN-CH_3O). The kinetics of LDL oxidation were determined by monitoring the absorbance of conjugated dienes at 234 nm, using the DU800 spectrophotometer at 4-minute intervals at 37°C.

2.7. Cell cultures

Human umbilical vein endothelial cells were purchased from Lonza Walkersville. They were cultured in endothelial cell growth medium-2 and used for experiments at passage 4. The human monocytic cell line THP-1 was obtained from RIKEN Cell Bank (Ibaraki, Japan) and cultured in RPMI-1640, which was supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C and under 5% CO_2.

2.8. HUVEC-mediated LDL oxidation assay

The LDL was dialyzed against phosphate-buffered saline (PBS) at 4°C to remove EDTA. To initiate the endothelial cell–mediated LDL oxidation, HUVECs were treated with dialyzed LDL (at the final concentration of 100 μg protein/mL), in the absence or presence of 1 to 10 μg/mL PBE, for 24 hours in Ham F10 medium. After incubation, the medium was collected. Low-density lipoprotein oxidation was evaluated by measuring malondialdehyde (MDA) and by examining the electrophoretic mobility of LDL subjected to agarose gel electrophoresis. Malondialdehyde was measured by using TBARS assay [31]. The TBARS reagent (15% [wt/vol] trichloroacetic acid, 0.375% [wt/vol] thiobarbituric acid—0.25 N HCl) was added to the sample and heated for 10 minutes at 90°C. The absorbance of reactive products was measured at 535 nm. The results are expressed in terms of relative MDA content (nmol/mg-LDL protein), which was calculated using the extinction coefficient for MDA. To evaluate the change in the surface charge of LDL, electrophoresis was performed at 400 V for 25 minutes by using a rapid electrophoresis system; the gels were stained with CHO/Trig CONBO CH (Helena Laboratories, Saitama, Japan) [32].

2.9. Gene expression analysis in THP-1 cells

THP-1 cells were treated with 0 to 10 μg/mL PBE for 24 hours. Total cellular RNA was extracted using RNAiso Plus (Takara Bio, Shiga, Japan). First-strand complementary DNA was synthesized from the total RNA (2 μg) by using TaqMan High Capacity complementary DNA Reverse Transcription Kit (Life Technologies Japan, Tokyo). Real-time PCR was performed on an ABI 7300 cycler (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green PCR mix (Life Technologies Japan). The results are expressed as the copy number ratio of the target messenger RNA (mRNA) to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA. The primer sequences used for the analysis of GPx (*GPX1*), catalase (*CAT*), and HO-1 (*HMOX1*) are as described in Table.

2.10. Protein expression analysis in THP-1 cells

THP-1 cells were treated with 0 to 10 μg/mL PBE for the times indicated below. After treatment, we washed the THP-1 cells with ice-cold PBS. We then prepared total cell and nuclear extracts lysates by using a protein extraction reagent and NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermoscientific, Rockford, IL, USA), which contained 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Aliquots of cellular proteins were electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA). The membrane was probed with the corresponding primary antibodies: anti-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-catalase (AbFRONTIER, Seoul, Korea), anti–extracellular signal-regulated kinase (ERK) and anti-Akt (Cell Signaling Technology, Danvers, NA, USA), anti-HO-1 (Enzo Life Science, New York, NY, USA), anti-GPx (Assay Biotech, Sunnyvale, CA, USA), and anti-actin (Sigma-Aldrich); then followed by incubation with peroxidase-conjugated antirabbit or mouse IgG secondary antibodies. The detection of specific proteins was performed by enhanced chemiluminescence. All

Table – List of primer sequence

Gene	Sequence (5′ to 3′)
SOD1	Forward: CATCATCAATTTCGAGCAGA
	Reverse: GCCACACCATCTTTGTCAGCAG
CAT	Forward: TGACCAGGGCATCAAAAACC
	Reverse: CGGATTGCCATAGTCAGGATCTT
GPX1	Forward: CTTCGAAAGTGCGAGGTG
	Reverse: AAACTTCCAGGCAACATCGT
HMOX1	Forward: TCAACATCCAGCTCTTTGAGGA
	Reverse: AGTGTAAGGACCCATCGGAGAA
GAPDH	Forward: TGCACCACCAACTGCTTAGC
	Reverse: GGCATGGACTGTGGTCATGAG

signals were detected by the LAS-4000 system (Fujifilm, Tokyo, Japan) and analyzed by Multi Gauge version 3.0 software.

2.11. *Measurement of ROS production in THP-1 cells*

THP-1 cells were treated with 0 to 10 μg/mL PBE for 2 hours. We measured intracellular ROS production by using DCFH-DA as the fluorescent probe [33]. THP-1 cells were incubated with 20 μM DCFH-DA for 30 minutes, then treated with fresh media containing 1 to 10 μg/mL PBE for 2 hours. Fluorescent intensity was measured by a microplate reader (BioTek Instruments, Tokyo, Japan) at 485-nm excitation and 528-nm emission.

2.12. *Study design of PBE intake in human subjects*

The study group consisted of 10 healthy male volunteers who ranged in age from 32 to 52 years. The study was approved by the Ethics Committee of Ochanomizu University and conformed to the Declaration of Helsinki. All subjects gave their informed consent to participate in the study. After the subjects fasted for at least 12 hours, blood samples were collected between 8:00 and 9:00 AM. The subjects then ingested 1.0 g of PBE powder with a glass of water, and plasma samples were taken at 0.5, 1, 2, and 4 hours after consumption. Low-density lipoprotein was isolated and oxidized by adding AMVN-CH_3O, and lag time, TBARS, and LDL mobility were determined, as described in Sections 2.6 and 2.8. Peripheral blood mononuclear cells were isolated from the buffy coat separated by the centrifugation of the blood samples from the subjects [34]. The cells were carefully layered onto Histopaque-1077 (Sigma-Aldrich) and centrifuged at 1600 rpm for 30 minutes at 4°C. The PBMCs were then washed using 2 mM EDTA-PBS and ammonium chloride solution. *SOD1*, *GPX1*, and *CAT* mRNA expressions were measured by real-time PCR.

2.13. *Statistical analyses*

All data are presented as means ± SD. Comparisons between treatment groups were performed using 1-way analysis of variance (ANOVA) with the post hoc test. The within-subject change from baseline during postprandial period was analyzed with Dunnett test, after repeated-measures 1-way ANOVA. Differences were considered significant when $P < .05$. The statistical analyses were performed using the GraphPad Prism 5 software package (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. *Total polyphenol content and radical scavenging activity of PBE*

The total polyphenol content of the PBE powder was 75.7% (wt/wt). Pine bark extract displayed DPPH radical scavenging ability (EC_{50} value was 4.0 μg/mL).

3.2. *PBE prevents LDL oxidation in vitro*

Fig. 1A shows that the PBE significantly prolonged the LDL oxidation lag time compared with the solvent control (0.5 μg/mL: 39 ± 4 minutes; 1.25 μg/mL: 63 ± 4 minutes; 2.5 μg/mL; 103 ± 5 minutes; and control: 18 ± 2 minutes), indicating an increased resistance to oxidation of the LDL.

We also examined the inhibitory effect of PBE on LDL oxidation mediated by endothelial cells. Low-density lipoprotein was incubated with HUVEC in Ham F10 medium (which contains Fe^{2+} and Cu^{2+}), in the presence or absence of PBE. The interaction of LDL with the HUVEC significantly increased TBARS production (data not shown), whereas PBE significantly inhibited TBARS production ($P < .001$; Fig. 1B). We also evaluated the change in the surface charge of LDL by agarose gel electrophoresis. The oxidation of LDL could be monitored by comparing the relative electrophoresis mobility of unmodified and modified LDL. Pine bark extract suppressed the negative charge of the LDL particles that resulted from HUVEC incubation, indicating the inhibition of apolipoprotein B100 modification ($P < .001$; Fig. 1C).

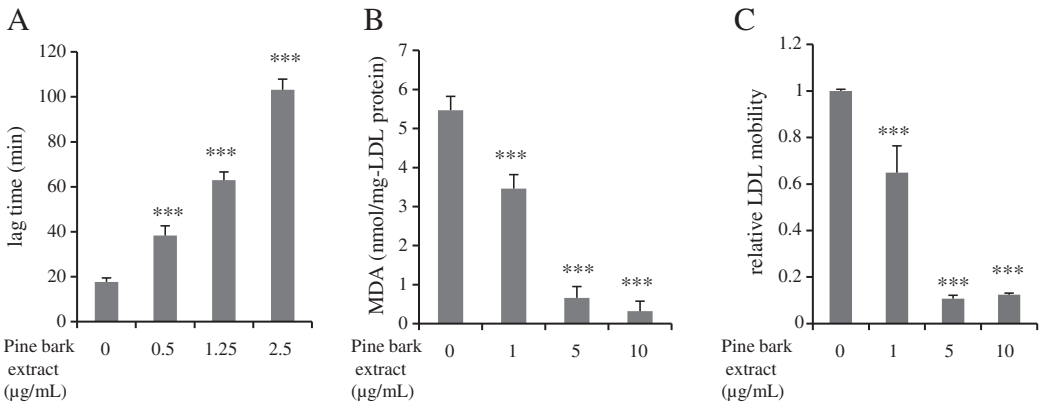

Fig. 1 – Effects of PBE on LDL oxidation in vitro LDL (70 μg/mL protein) was incubated with 400 μM AMVN-CH_3O in the absence or presence of PBE at 37°C. A, The lag time for starting LDL oxidation was defined as the time interval between the initiation and intercept of the 2 tangents drawn to the lag and propagation phase of the absorbance curve at 234 nm. Low-density lipoprotein was incubated with HUVECs in Ham F10 medium in the presence or absence of PBE for 24 hours. The lipid oxidation product was assessed by a TBARS assay (B), and the LDL mobility was measured by agarose gel electrophoresis (C). Values are means ± SD (n = 4). ***$P < .001$, compared with untreated control by Tukey test after 1-way ANOVA.

3.3. *PBE induces antioxidant enzyme expression in THP-1 cells*

To clarify the effect on antioxidant enzyme expression in monocytic cells, we treated THP-1 cells with PBE. As shown in Fig. 2A, PBE significantly increased the mRNA expressions of *GPX1*, *CAT*, and *HMOX1* at 10 μg/mL (by 1.3 ± 0.2-fold, 1.5 ± 0.3-fold, and 1.4 ± 0.3-fold, respectively). These data correlated with the increases in the protein expression (Fig. 2B).

3.4. *Regulatory effects of PBE on Nrf2, MAPK, and Akt in THP-1 cells*

Nrf2 plays a key role in the regulation of antioxidant enzymes. To examine whether PBE induced Nrf2 translocation, the expression levels of Nrf2 in both the cytosolic and nuclear proteins were analyzed by Western blotting. Pine bark extract induced the accumulation of Nrf2 in the nuclear protein at 2 hours (Fig. 3A). To examine whether the Nrf2 activation by PBE is associated with upstream kinase action, we examined the activation of Akt and MAPK. Pine bark extract rapidly stimulated the phosphorylation of Akt and ERK, with peak induction detected at 30 minutes (Fig. 3B).

3.5. *PBE decreases ROS production in THP-1 cells*

To examine the oxidative stress condition, we measured the intracellular ROS production levels. Levels of ROS were significantly decreased by PBE in a dose-dependent manner (Fig. 4).

3.6. *Antioxidant effects of PBE intake in human subjects*

Ten healthy adult subjects consumed 1.0 g of PBE. As shown in Fig. 5A, the LDL oxidation lag time was prolonged at 0.5, 1, 2, and 4 hours after the ingestion of PBE compared with that before intake ($P < .05$, $P < .05$, $P < .05$, and $P < .01$, respectively). The TBARS production and LDL mobility were not significantly changed (Fig. 5B, C). These results show that PBE could reduce LDL oxidizability in human subjects as well as in vitro. Fig. 5D shows the changes in antioxidant enzyme mRNA expression

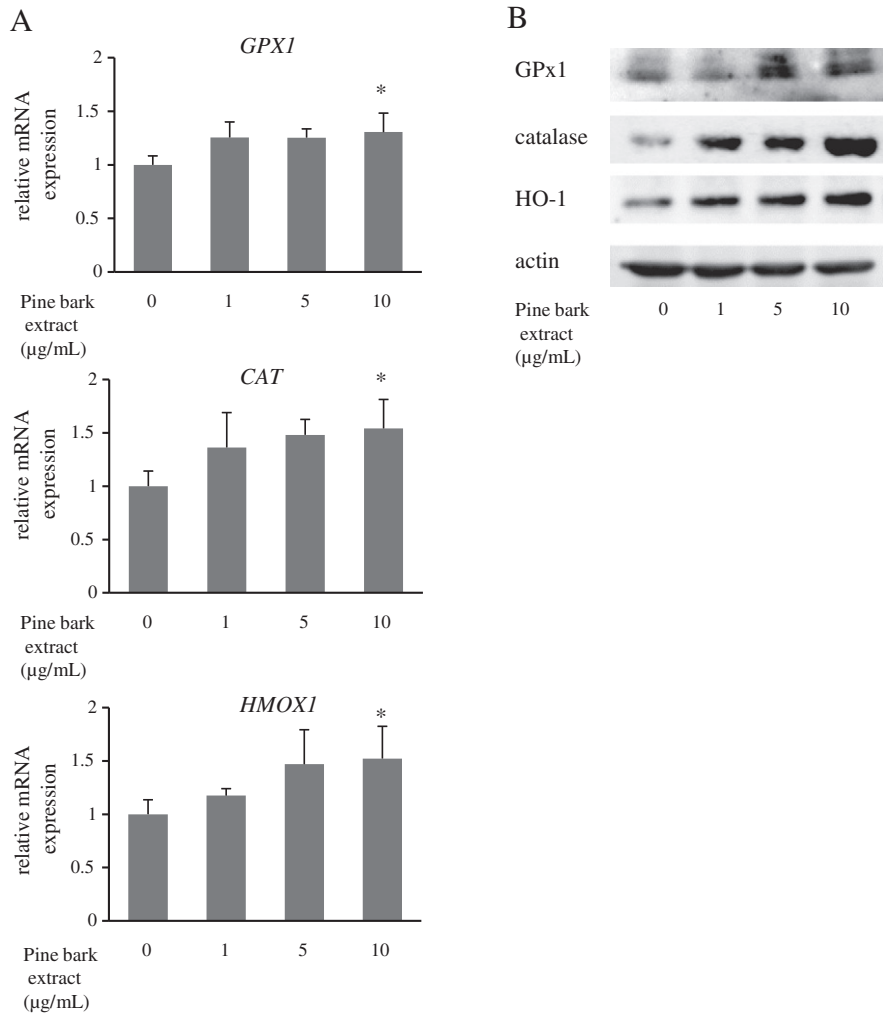

Fig. 2 – Effects of PBE on antioxidant enzyme expressions. THP-1 cells were incubated with 0 to 10 μg/mL PBE for 24 hours. *GPX1*, *CAT*, and *HMOX1* mRNA expressions were measured by real-time reverse transcriptase PCR. A, Values are means ± SD (n = 4). *P < .05, compared with untreated control by Tukey test after 1-way ANOVA. Protein expressions were measured by Western blot assay. Equal loading of proteins was confirmed with total actin antibody. B, Representative blot experiments are shown.

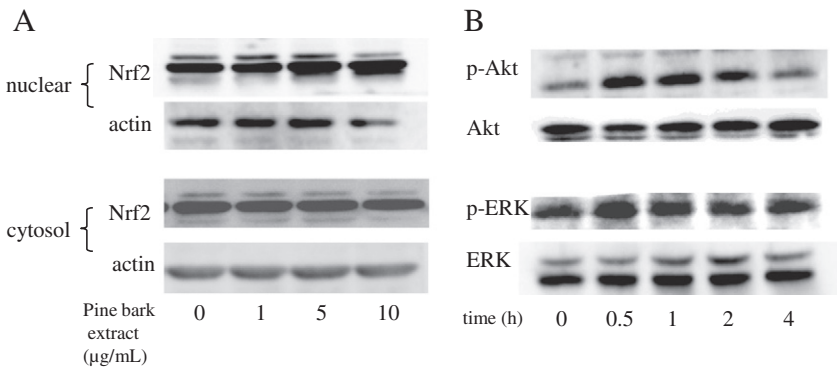

Fig. 3 – Effects of PBE on Nrf2 and upstream kinase activations. A, THP-1 cells were incubated with 0 to 10 μg/mL PBE for 2 hours, and the nuclear and cytoplasmic Nrf2 proteins were detected by Western blot analysis. B, After incubation with 10 μg/mL PBE for the indicated times, Akt and ERK activation was determined by Western blot with phospho-specific antibodies. Representative blot experiments are shown.

in PBMC between the before and after intakes of PBE. The PBE slightly increased the *SOD1* mRNA expression at 4 hours by 2.1 ± 2.4-fold, but the difference was not statistically significant (P = .17). The *GPX1* and *CAT* expressions were not significantly changed (*GPX1*: 1.0 ± 0.4 vs 1.5 ± 1.2 and *CAT*: 1.0 ± 1.3 vs 0.8 ± 0.7).

4. Discussion

The oxidative modification of LDL is known to play a critical role in the initiation and development of atherosclerosis. In the present study, we observed that PBE enhanced the antioxidant defense capacity of LDL and monocytes.

We first evaluated the antioxidant activity of this extract. The PBE contained polyphenols and showed DPPH radical scavenging ability. The major components of PBE are known to be monomers (eg, (+)-catechin, (–)-epicatechin), dimers (eg, proanthocyanidin B1), and oligomers [35,36]. Catechins and proanthocyanidins are reported to have free radical scavenging ability [37,38]. The oxidation of LDL is induced by various mechanisms, such as active metal ions, free radicals, and some pro-oxidant enzymes [39]. In the present study, PBE significantly prolonged the LDL lag time, reflecting the resistance of free radical–induced LDL lipid peroxidation. Pine bark extract also inhibited endothelial cell–induced LDL oxidation. Some polyphenols (eg, epicatechin and oligomeric procyanidin) have been reported to inhibit 15-lipoxygenase–mediated LDL lipid peroxidation [40,41]. These data suggest that PBE could prevent endothelial-induced LDL oxidation due to a radical scavenging and/or inhibitory effect against pro-oxidant enzymes actions.

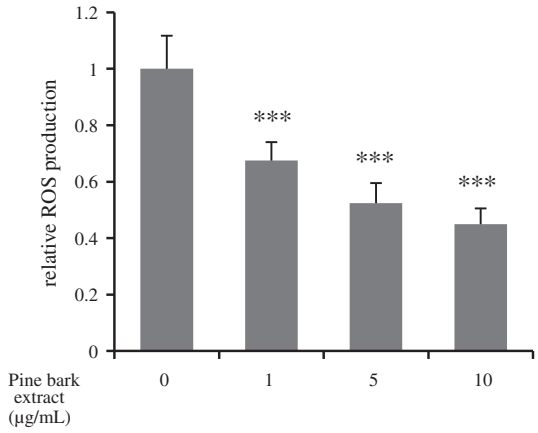

Fig. 4 – Effects of PBE on ROS production. THP-1 cells were incubated with 0 to 10 μg/mL PBE for 2 hours, and then intracellular ROS levels were measured by using DCFH-DA (excitation 500 nm/emission 536 nm). Values are means ± SD (n = 4). ***P < .01, compared with untreated control by Tukey test after 1-way ANOVA.

One of the major cellular antioxidant responses is the induction of antioxidant enzymes through the Nrf2-Keap1 pathway. Nrf2 plays a crucial role in inducing the genes of antioxidant/phase 2 enzymes [42,43]. Nrf2 is bound to the cytosolic repressor protein Keap1, which leads to Nrf2 ubiquitination and proteasome degradation under basal conditions. Once the Nrf2-Keap1 pathway is activated by inducers, Nrf2 is separated from Keap1, thus leading to stabilization and accumulation in the nucleus and, subsequently, to an increase in the expressions of antioxidant response element–containing genes, such as GPx, catalase, and HO-1 [16,19,43]. The Nrf2-Keap1 pathway can be regulated by various endogenous stresses (eg, oxidative stress and shear stress) and endogenous chemopreventive agents (eg, nitric oxide and phytochemicals) [16,19]. We demonstrated that PBE activated Nrf2 and increased the expressions of *GPX1*, *CAT*, and *HMOX1* in human monocytic THP-1 cells. The important role that Nrf2 plays in the chemical induction of antioxidant enzymes, including GPx and catalase, is reported in a study using cardiac fibroblasts from $Nrf2^{-/-}$ mice [44]. Another group stated that epigallocatechin increased the HO-1 expression, which was significantly canceled by Nrf2 silencing, in THP-1 cells [45]. Several kinases (eg, MAPK and Akt/PI3K pathway) are also implicated in the activation of Nrf2 [19,46,47]. Procyanidins are also noted to induce Nrf2 accumulation with the activation of all 3 MAPKs (ERK, JNK, and p38), as well as Akt, which was attenuated by a PI3K

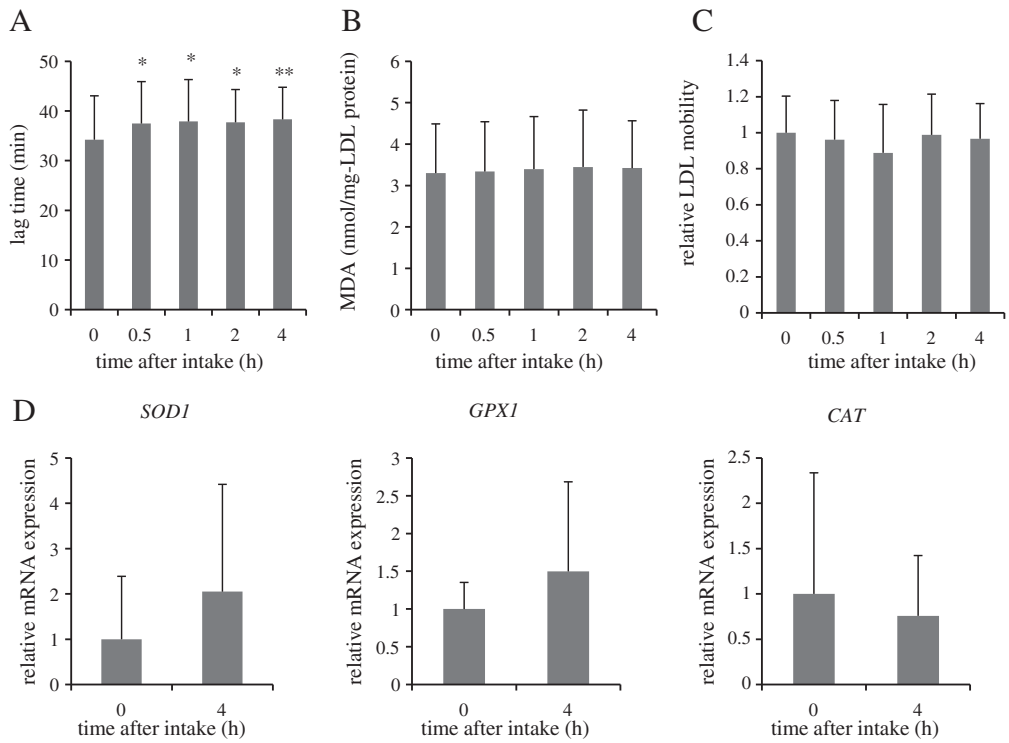

Fig. 5 – Pine bark extract up-regulated antioxidant defense capacity in human subjects. After overnight fasting, 10 healthy volunteers consumed 1.0 g of PBE. Blood samples were collected before and at 0.5, 1, 2, and 4 hours after intake. The LDL oxidizability was measured by a lag time assay (A), TBARS assay (B), and agarose gel electrophoresis (C). D, Total RNA was extracted from isolated PBMCs, and *SOD1*, *GPX1*, and *CAT* mRNA expressions were measured by real-time reverse transcriptase PCR. Values are means ± SD (n = 8-10). **P < .01, *P < .05, compared with 0 hours by Dunnett test after repeated-measures 1-way ANOVA.

inhibitor and a p38 inhibitor but not an ERK inhibitor in human hepatic cells [48]. Using human colonic cells, Rodriguez-Ramiro et al [49] reported that procyanidin B2 increased Nrf2 translocation via ERK and p38 activation. Because Nrf2 is a transcriptional activator that plays a critical role in the response to oxidative stress, we examined the effect of PBE on intracellular ROS status. Pine bark extract decreased the ROS level in THP-1 cells, suggesting that the induction of antioxidant enzymes by PBE might not be mediated by ROS generation. Our results suggest a unique pathway by which PBE might induce antioxidant enzyme expression through Nrf2 activation mediated by ERK and Akt, but not accompanied by ROS generation.

The present study also revealed that consumption of PBE could decrease the susceptibility of LDL to oxidation in human subjects. Plasmas are suggested to be potential carriers of polyphenols [50]. Grimm et al [51] demonstrated that catechin, ferulic acid, and caffeic acid were detectable in human plasma from 0.5 to 14 hours after the administration of 300 mg of PBE as a single dose; the maximum plasma concentration of catechin was approx. 100 ng/mL at 4 hours after administration. We previously reported that the intake of red perillas or sweet potato leaves, both of which are polyphenol-rich leaf vegetables, showed an inhibitory effect on LDL oxidation at 1 hour after intake [52,53]. Although not directly investigated in our experiments, a previous study reported that plasma total phenol levels were significantly increased to 5.67 mg phenol equivalent/L after 3 weeks of PBE supplementation [27]. Thus, PBE intake may be associated with an increase in plasma polyphenol levels, and the polyphenols in plasma may exert an inhibitory effect against LDL oxidation.

Peripheral blood mononuclear cells are populations of immune cells (such as monocytes and lymphocytes) that are known to play important roles in oxidative stress and inflammation. These cells are often used to investigate the biological effects of dietary intervention [20]. The *SOD1* mRNA expression tended to be higher at 4 hours after PBE intake, as compared with baseline. Oral phenolic acid administration increased the cardiac *SOD1*, *GPX1*, and *CAT* mRNA expressions and activities in rats [54], a finding that may support our hypothesis in that PBE might exert antioxidant activities due partly to an induction of antioxidant enzyme expressions.

Although this study is limited in that the human experiments were performed as a self-controlled design and its sample size was rather small to detect treatment differences in the mRNA expressions in PBMC, the overall results are promising.

The study results indicate that PBE enhanced the antioxidant defense capacity of LDL and monocytes, thus suggesting that PBE could be effective in the reduction of atherosclerosis risk factors associated with oxidative stress. However, measurements of the plasma polyphenol level or antioxidant enzymes activity following intake of PBE are be needed to determine the direct effect of PBE.

Acknowledgment

This study was supported, in part, by JSPS KAKENHI (Grant No. 23240104) and by Toyo Shinyaku Co Ltd. We thank Robert Matera for editing this manuscript. We also thank the clinical study volunteers and all members of the Kondo Laboratory for the critical discussions.

REFERENCES

[1] Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 1989;320:915–24.
[2] Pashkow FJ. Oxidative stress and inflammation in heart disease: do antioxidants have a role in treatment and/or prevention? Int J Inflamm 2011;2011:514623.
[3] Kaplan M, Aviram M. Oxidized low density lipoprotein: atherogenic and proinflammatory characteristics during macrophage foam cell formation. An inhibitory role for nutritional antioxidants and serum paraoxonase. Clin Chem Lab Med 1999;37:777–87.
[4] Burchardt P, Zurawski J, Zuchowski B, Kubacki T, Murawa D, Wiktorowicz K, et al. Low-density lipoprotein, its susceptibility to oxidation and the role of lipoprotein-associated phospholipase A2 and carboxyl ester lipase lipases in atherosclerotic plaque formation. Arch Med Sci 2013;9:151–8.
[5] Miura S, Watanabe J, Sano M, Tomita T, Osawa T, Hara Y, et al. Effects of various natural antioxidants on the Cu(2+)-mediated oxidative modification of low density lipoprotein. Biol Pharm Bull 1995;18:1–4.
[6] Thuong PT, Pokharel YR, Lee MY, Kim SK, Bae K, Su ND, et al. Dual anti-oxidative effects of fraxetin isolated from *Fraxinus rhinchophylla*. Biol Pharm Bull 2009;32:1527–32.
[7] Yoshida HIT, Hosoai H. Inhibitory effect of tea flavonoids on the ability of cells to oxidize low density lipoprotein. Biochem Pharmacol 1999;58:1695–703.
[8] Kondo K, Hirano R, Matsumoto A, Igarashi O, Itakura H. Inhibition of LDL oxidation by cocoa. Lancet 1996;348:1514.
[9] Kalkan Yildirim H, Delen Akcay Y, Guvenc U, Yildirim Sozmen E. Protection capacity against low-density lipoprotein oxidation and antioxidant potential of some organic and non-organic wines. Int J Food Sci Nutr 2004;55:351–62.
[10] Hertog MG, Feskens EJ, Kromhout D. Antioxidant flavonols and coronary heart disease risk. Lancet 1997;349:699.
[11] Huxley RR, Neil HA. The relation between dietary flavonol intake and coronary heart disease mortality: a meta-analysis of prospective cohort studies. Eur J Clin Nutr 2003;57:904–8.
[12] Augusti PR, Ruviaro AR, Quatrin A, Somacal S, Conterato GM, Vicentini JT, et al. Imbalance in superoxide dismutase/thioredoxin reductase activities in hypercholesterolemic subjects: relationship with low density lipoprotein oxidation. Lipids Health Dis 2012;11:79.
[13] Uto-Kondo H, Ayaori M, Kishimoto Y, Adachi T, Takiguchi S, Yakushiji E, et al. Consumption of polyphenol-rich juar tea increases endothelium-bound extracellular superoxide dismutase levels in men with metabolic syndrome: link with LDL oxidizability. Int J Food Sci Nutr 2013;64:407–14.
[14] Yang H, Roberts LJ, Shi MJ, Zhou LC, Ballard BR, Richardson A, et al. Retardation of atherosclerosis by overexpression of catalase or both Cu/Zn-superoxide dismutase and catalase in mice lacking apolipoprotein E. Circ Res 2004;95:1075–81.
[15] Juan SH, Lee TS, Tseng KW, Liou JY, Shyue SK, Wu KK, et al. Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E–deficient mice. Circulation 2001;104:1519–25.
[16] Baird L, Dinkova-Kostova AT. The cytoprotective role of the Keap1-Nrf2 pathway. Arch Toxicol 2011;85:241–72.
[17] Cardozo LF, Pedruzzi LM, Stenvinkel P, Stockler-Pinto MB, Daleprane JB, Leite Jr M, et al. Nutritional strategies to modulate inflammation and oxidative stress pathways via activation of the master antioxidant switch Nrf2. Biochimie 2013;95:1525–33.
[18] Han X, Shen T, Lou H. Dietary polyphenols and their biological significance. Int J Mol Sci 2007;8:950–88.
[19] Surh YJ, Kundu JK, Na HK. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. Planta Med 2008;74:1526–39.
[20] de Mello VD, Kolehmanien M, Schwab U, Pulkkinen L, Uusitupa M. Gene expression of peripheral blood mononuclear cells as a tool in dietary intervention studies: what do we know so far? Mol Nutr Food Res 2012;56:1160–72.
[21] Furumura M, Sato N, Kusaba N, Takagaki K, Nakayama J. Oral administration of French maritime pine bark extract (Flavangenol((R))) improves clinical symptoms in photoaged facial skin. Clin Interv Aging 2012;7:275–86.
[22] Drieling RL, Gardner CD, Ma J, Ahn DK, Stafford RS. No beneficial effects of pine bark extract on cardiovascular disease risk factors. Arch Intern Med 2010;170:1541–7.
[23] Ohkita M, Kiso Y, Matsumura Y. Pharmacology in health foods: improvement of vascular endothelial function by French maritime pine bark extract (Flavangenol). J Pharmacol Sci 2011;115:461–5.
[24] Guo Q, Zhao B, Packer L. Electron spin resonance study of free radicals formed from a procyanidin-rich pine (*Pinus maritima*) bark extract, pycnogenol. Free Radic Biol Med 1999;27:1308–12.
[25] Peng Q, Wei Z, Lau BH. Pycnogenol inhibits tumor necrosis factor-alpha–induced nuclear factor kappa B activation and adhesion molecule expression in human vascular endothelial cells. Cell Mol Life Sci 2000;57:834–41.
[26] Golanski J, Muchova J, Golanski R, Durackova Z, Markuszewski L, Watala C. Does pycnogenol intensify the efficacy of acetylsalicylic acid in the inhibition of platelet function? In vitro experience. Postepy Hig Med Dosw (Online) 2006;60:316–21.
[27] Devaraj S, Vega-Lopez S, Kaul N, Schonlau F, Rohdewald P, Jialal I. Supplementation with a pine bark extract rich in polyphenols increases plasma antioxidant capacity and alters the plasma lipoprotein profile. Lipids 2002;37:931–4.
[28] Enseleit F, Sudano I, Periat D, Winnik S, Wolfrum M, Flammer AJ, et al. Effects of Pycnogenol on endothelial function in patients with stable coronary artery disease: a double-blind, randomized, placebo-controlled, cross-over study. Eur Heart J 2012;33:1589–97.
[29] Iizuka M, Tani M, Kishimoto Y, Saita E, Toyozaki M, Kondo K. Inhibitory effects of balsamic vinegar on LDL oxidation and lipid accumulation in THP-1 macrophages. J Nutr Sci Vitaminol (Tokyo) 2010;56:421–7.
[30] Hirano R, Kondo K, Iwamoto T, Igarashi O, Itakura H. Effects of antioxidants on the oxidative susceptibility of low-density lipoprotein. J Nutr Sci Vitaminol (Tokyo) 1997;43:435–44.
[31] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302–10.
[32] Yoshida H, Sasaki K, Namiki Y, Sato N, Tada N. Edaravone, a novel radical scavenger, inhibits oxidative modification of low-density lipoprotein (LDL) and reverses oxidized LDL-mediated reduction in the expression of endothelial nitric oxide synthase. Atherosclerosis 2005;179:97–102.
[33] Rosenkranz AR, Schmaldienst S, Stuhlmeier KM, Chen W, Knapp W, Zlabinger GJ. A microplate assay for the detection of oxidative products using 2′,7′-dichlorofluorescin-diacetate. J Immunol Methods 1992;156:39–45.

[34] Nantz MP, Rowe CA, Muller C, Creasy R, Colee J, Khoo C, et al. Consumption of cranberry polyphenols enhances human gammadelta-T cell proliferation and reduces the number of symptoms associated with colds and influenza: a randomized, placebo-controlled intervention study. Nutr J 2013;12:161.
[35] Tsubata M, Takagaki K, Hirano S, Iwatani K, Abe C. Effects of flavangenol, an extract of French maritime pine bark on collagen-induced arthritis in rats. J Nutr Sci Vitaminol (Tokyo) 2011;57:251–7.
[36] Yoshida A, Yoshino F, Tsubata M, Ikeguchi M, Nakamura T, Lee MC. Direct assessment by electron spin resonance spectroscopy of the antioxidant effects of French maritime pine bark extract in the maxillofacial region of hairless mice. J Clin Biochem Nutr 2011;49:79–86.
[37] da Silva JMR, Darmon N, Fernandez Y, Mitjavila S. Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. J Agric Food Chem 1991;39: 1549–52.
[38] Arimboor R, Arumughan C. Effect of polymerization on antioxidant and xanthine oxidase inhibitory potential of sea buckthorn (*H. rhamnoides*) proanthocyanidins. J Food Sci 2012; 77:C1036–41.
[39] Yoshida H, Kisugi R. Mechanisms of LDL oxidation. Clin Chim Acta 2010;411:1875–82.
[40] da Silva EL, Abdalla DS, Terao J. Inhibitory effect of flavonoids on low-density lipoprotein peroxidation catalyzed by mammalian 15-lipoxygenase. IUBMB Life 2000;49:289–95.
[41] Schewe T, Sadik C, Klotz LO, Yoshimoto T, Kuhn H, Sies H. Polyphenols of cocoa: inhibition of mammalian 15-lipoxygenase. Biol Chem 2001;382:1687–96.
[42] Gupte AA, Lyon CJ, Hsueh WA. Nuclear factor (erythroid-derived 2)-like-2 factor (Nrf2), a key regulator of the antioxidant response to protect against atherosclerosis and nonalcoholic steatohepatitis. Curr Diab Rep 2013;13:362–71.
[43] Hybertson BM, Gao B, Bose SK, McCord JM. Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation. Mol Aspects Med 2011;32:234–46.
[44] Zhu H, Itoh K, Yamamoto M, Zweier JL, Li Y. Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. FEBS Lett 2005;579:3029–36.
[45] Ogborne RM, Rushworth SA, O'Connell MA. Epigallocatechin activates haem oxygenase-1 expression via protein kinase Cdelta and Nrf2. Biochem Biophys Res Commun 2008;373: 584–8.
[46] Paine A, Eiz-Vesper B, Blasczyk R, Immenschuh S. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. Biochem Pharmacol 2010;80:1895–903.
[47] Nguyen T, Yang CS, Pickett CB. The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. Free Radic Biol Med 2004;37:433–41.
[48] Bak MJ, Jun M, Jeong WS. Procyanidins from wild grape (*Vitis amurensis*) seeds regulate ARE-mediated enzyme expression via Nrf2 coupled with p38 and PI3K/Akt pathway in HepG2 cells. Int J Mol Sci 2012;13:801–18.
[49] Rodriguez-Ramiro I, Ramos S, Bravo L, Goya L, Martin MA. Procyanidin B2 induces Nrf2 translocation and glutathione S-transferase P1 expression via ERKs and p38-MAPK pathways and protect human colonic cells against oxidative stress. Eur J Nutr 2012;51:881–92.
[50] Carbonneau MA, Leger CL, Monnier L, Bonnet C, Michel F, Fouret G, et al. Supplementation with wine phenolic compounds increases the antioxidant capacity of plasma and vitamin E of low-density lipoprotein without changing the lipoprotein Cu(2+)-oxidizability: possible explanation by phenolic location. Eur J Clin Nutr 1997;51:682–90.
[51] Grimm T, Skrabala R, Chovanova Z, Muchova J, Sumegova K, Liptakova A, et al. Single and multiple dose pharmacokinetics of maritime pine bark extract (pycnogenol) after oral administration to healthy volunteers. BMC Clin Pharmacol 2006;6:4.
[52] Saita EKY, Tani M, Iizuka M, Toyozaki M, Sugihara N, Kondo K. Antioxidant activities of *Perilla frutescens* against low-density lipoprotein oxidation in vitro and in human subjects. J Oleo Sci 2012;61:113–20.
[53] Nagai M, Tani M, Kishimoto Y, Iizuka M, Saita E, Toyozaki M, et al. Sweet potato (*Ipomoea batatas* L.) leaves suppressed oxidation of low density lipoprotein (LDL) in vitro and in human subjects. J Clin Biochem Nutr 2011;48:203–8.
[54] Yeh CT, Ching LC, Yen GC. Inducing gene expression of cardiac antioxidant enzymes by dietary phenolic acids in rats. J Nutr Biochem 2009;20:163–71.