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Protective effects of pu-erh tea on LDL oxidation and nitric oxide generation in macrophage cells

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Abstract

Effects of water extract of pu-erh tea (WEPT) on low density of lipoprotein (LDL) oxidation and their regulation of nitric oxide in macrophage RAW 264.7 cells were determined. The results showed that WEPT significantly scavenged H_2O_2 in a concentration-dependent manner. In the range of 0–0.1 mg/mL, the inhibitory action on LDL oxidation increased with increasing concentration of WEPT. In addition, WEPT scavenged $O_2^{\bullet -}$ and inhibited the activity of xanthine oxidase, suggested that WEPT showed effectively inhibitory effect on oxidative stress. WEPT at 200 μg/mL not only increased the glutathione (GSH) content by 41.5-fold but also enhanced activity of glutathione peroxidase (GPX), glutathione reductase (GR), glutathione transferase (GST) and catalase (CAT) in 3T3 cells by 1.8-, 2.6-, 10.9- and 4.3-fold compared to the control, respectively, revealing that WEPT may positively modulate the GSH and antioxidant enzyme system. Moreover, WEPT scavenged NO generated by sodium nitroprusside (SNP). The expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-activated macrophage RAW 264.7 cells was inhibited by WEPT. Reverse transcription-polymerase chain reaction (RT-PCR) analysis indicated that the iNOS mRNA expression was suppressed by WEPT. Furthermore, the down-regulated degradation of IκB-α by WEPT was found, indicating that WEPT suppressed iNOS enzyme expression as a result of preventing nuclear factor-kappa B (NF-κB) activation. The HPLC analyses showed that gallic acid, epicatechin and caffeine, which are polyphenolics with bioactive action, were found in WEPT. Overall, these findings suggest that pu-erh tea may play a crucial role in preventing such oxidation-related diseases as atherosclerosis and other types of vascular diseases.

Keywords: Pu-erh tea; LDL oxidation; Reactive oxygen species; Nitric oxide; iNOS expression; Gallic acid; Epicatechin; Caffeine

1. Introduction

The low-density lipoprotein (LDL) which consists of ~25% apo B-100 protein and approximately 75% lipid consisting almost entirely of cholesterol esters and some triglycerides is the major cholesterol-carrying lipoprotein in plasma (Kalyanaraman, 1995). The polyunsaturated fatty acids (PUFAs) in LDL are linoleate associated with cholsteryl ester, which are very susceptible to oxidative damage. Rapidly accumulating evidence supports that oxidation of LDL plays a crucial role in the development of atherosclerosis in human (Stampferh & Rimm, 1994). Connor and Nalebuff (1995) noted that the protective action of mechanism on LDL oxidation by antioxidants is free radicals scavenging and iron(II) chelating capacity. Thus, the best way to prevent the formation of a fatty steak of atherosclerosis at initial stage is to inhibit LDL oxidation. Epidemiological studies have shown that diets rich antioxidants are associated with lower risk of cardiovascular diseases (Ness & Powles, 1997). Therefore, studies investigating inhibitory effects on LDL oxidation by dietary intake of antioxidative compounds and assay the natural antioxidant source have attracted much attention.

The reactive nitrogen species (RNS) such as nitric oxide (NO), peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2) play an important role in inflammatory process

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(Connor & Nalebuff, 1995). Under inflammatory conditions, macrophages may greatly produce both levels of NO and superoxide, which rapidly react with each other to form peroxynitrite. Subsequently, peroxynitrite oxidizes LDL, a key process in atherosclerosis (Pannala, Rice-Evans, Halliwell, & Singh, 1997). Also, DNA undergoes peroxynitrite-induced base modification, which can lead to strand breakage (Spencer et al., 1996). Under physiological conditions, mass superoxide production depends on some metabolic enzyme activities (e.g., xanthine oxidase and NADPH oxidase). And, it is well known that NO is generated by a family of isoforms of NO synthase in cells. One is a constitutive isoform (cNOS), which is subdivided into neuronal (nNOS) and endothelial (eNOS). Another is an inducible isoform (inducible NO synthase, iNOS) that execute mass NO generation (Kim, Murakami, Nakamura, & Ohigashi, 1998). NO produced by eNOS and iNOS has different effects in cardiovascular diseases; eNOS produced small quantity of NO in atherosclerotic lesions and played a positively protective role, and the large levels of NO produced by iNOS contributes to vascular injury (Kim et al., 2001). In other words, high amounts of superoxide and NO are potentially cytotoxic, possessing damaging menace to the surrounding cells and tissues indiscriminately by itself or by formation of $ONOO^-$. Thus, the inhibition of superoxide and NO generation, respectively, and iNOS expression may be associated with prevention of vascular diseases.

The Chinese people consume pu-erh tea, produced mainly in the Yunnan province of China, in a large amount. Crude green tea is used as raw material for preparation of the pu-erh tea after fermentation for a long time. The microorganism *Aspergillus niger* is often found in pu-erh tea. As for the quality and taste of pu-erh tea, it is believed that the longer the preservation period, the better the quality and taste. With the special taste and flavor, tea is one of the most popular drinks and it has been known for a long time as a drug preventing many diseases including cardiovascular diseases (Tijburg, Mattern, Folts, Weisgerber, & Katan, 1997) and cancer (Chung, Schwartz, Herzog, & Yang, 2003). The literature is replete with reports of biological effects in tea and tea-derived products. However, data regarding the biological action of pu-erh tea are limited in literature. In our previous studies, pu-erh tea demonstrated significant inhibitions on lipid and non-lipid oxidation similar to that of green tea extract, oolong tea extract and black tea extract (Duh, Yen, Yen, Wang, & Chang, 2004). In addition, we found that pu-erh tea directly scavenging NO radicals by pu-erh tea contributed to suppress NO production in RAW 264.7 cells (Duh et al., 2004). In addition, Miura et al. (1995) noted that the levels of plasma cholesterol ester and triglyceride in the plasma of rats given pu-erh tea were significantly reduced. Apart from these observations, little information is available about the biological activity of pu-erh tea. Although pu-erh tea may have potential as an antioxidant and as a NO scavenger, the investigations of protective effects of pu-erh tea on LDL oxidation and the regulating mechanism of pu-erh tea on NO generation have not been clearly elucidated. Thus, the aims of the present study were to assay the protective effects of water extract pu-erh tea (WEPT) on LDL oxidation and of RNS in macrophages.

2. Materials and methods

2.1. Chemicals

Horseradish peroxidase (HRP), butylated hydroxytoluene (BHT), xanthine oxidase, hypoxanthine, NADH, phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), 1-chloro-2,4-dinitrobenzene (CDNB), thiobarbituric acid (TBA), lipopolysaccharide (LPS, *Escherichia coli* 0127: B8) and sodium nitroprusside were purchased from Sigma (St. Louis, MO, USA). Ferrozine, Trolox (a water-soluble analog of vitamin E) and dinitrophenylhydrazine (DNPH) were purchased from Aldrich (St. Louis).

2.2. Sample preparation

Pu-erh tea (20 g) was extracted with boiling water (200 mL) for 5 min, and filtrate was freeze-dried. The final dehydrated WEPT powder (2.4 g) was then dissolved in phosphate buffer saline. This sample was named as water extracts of pu-erh tea (WEPT). The contents of gallic acid, epicatechin (EC) and caffeine in WEPT were determined by high-performance liquid chromatography (HPLC) and were 16.48, 1.80 and 76.13 μg/g, respectively.

2.3. Assay of scavenging activity on hydrogen peroxide (H_2O_2)

The scavenging activity on H_2O_2 was analyzed using H_2O_2-dependent, HRP-mediated oxidation of phenol red system according to a previously described method (Fowler, Daroszewska, & Ingold, 2003). H_2O_2 (2 mmol/L) and various concentrations of WEPT in phosphate buffer (0.05 M KH_2PO_4, pH 7.4, 5 mmol/L dextrose) were incubated at 25 °C for 5 min. After incubation, 0.1 mL phenol red (15 mmol/L) and 0.1 mL HRP (100 unit/mL) was added for 10 min, and the absorbance was read at 610 nm.

2.4. Activity of WEPT against low-density lipoprotein (LDL) oxidation

Human LDL ($d = 1.02$–1.06 g/mL) was prepared from fasting plasma, routinely pooled from healthy normolipemia individuals. Lipoproteins were isolated by sequential preparative ultra-centrifugation and dialyzed overnight as previously described (Viana et al., 1996). Freshly prepared native LDL (0.1 mg/mL) was treated with 10 μM $CuSO_4$ and in the presence of WEPT or not for up to 24 h at 37 °C and oxidation was stopped by addition of BHT. LDL oxidation was analyzed by TBA-reactive substance assay as