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Upregulation of erythrocyte ascorbate free radical reductase by tea catechins: Correlation with their antioxidant properties

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ABSTRACT

Tea catechins have been shown to possess a variety of health promoting effects, most of these effects have been attributed to their strong antioxidant property. Ascorbate (ASC) is the primary antioxidant in plasma and human erythrocytes play a vital role in regereneration of ASC in blood plasma by reduction of ascorbate free radical (AFR) into ASC by AFR reductase, a transmembrane enzyme. In the present study we report that tea catechins; EGCG, ECG, EGC and EC driven activation of erythrocyte AFR reductase. The efficiency of tea catechins to activate AFR reductase has a significant positive correlation with the antioxidant activities of these compounds, measured by DPPH• scavenging and FRAP assays. Since AFR reductase along with other transmembrane oxidoreductases, is reported to act as compensatory/protective mechanism that operates to minimize the extent of oxidative stress, activation of this enzyme by tea catechins may be a mechanism for these compounds to elicit antioxidant effect *in vivo*.

1. Introduction

Tea catechins, belonging to flavonoid group of polyphenols are the major components of tea, derived from the leaves of *Camellia sinensis* plant. Epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC) are quantitatively most important catechins in green tea (Fig. 1). Epidemiological studies and clinical observations have shown chemopreventive and other therapeutic potential of tea catechins in toxicity and diseases (Higdon & Frei, 2003; Khan & Mukhtar, 2008). Tea catechins are known to elicit anti-oxidant, neuroprotective, vasorelaxant and hypoglycaemic activities (Cabrera, Artacho, & Giménez, 2006; Khan & Mukhtar, 2008; Rizvi, Zaid, Anis, & Mishra, 2005), however the exact mechanisms underlying these properties of catechins remain speculative.

Ascorbic acid (ASC) is the primary antioxidant present in plasma, contributing in first line of protection against oxidative injury. In the presence of oxidants, ASC is oxidized first to ascorbate free radical (AFR) and then to dehydroascorbate (DHA), which is unstable and undergoes irreversible hydrolysis to 2, 3-diketo-L-gulonic acid (Fig. 2), resulting in decreased level of the vitamin in the body (Harrison & May, 2009). ASC can recycle α-tocopherol in low-density lipoprotein (LDL) in the face of an oxidant stress and thus affords protection against oxidation (Hemila & Kaprio, 2009; Jialal, Vega, & Grundy, 1990).

In addition to antioxidant property, ASC also serves as a cofactor in several important enzyme reactions including those involved in the synthesis of catecholamines, carnitine, cholesterol, amino acids, and certain peptide hormones (Harrison & May, 2009). In view of such a vital role in physiology the urinary loss of this vitamin makes it necessary to maintain high intracellular ASC concentration and to have efficient systems for recycling oxidized form of the vitamin to reduced form.

It is reported that AFR may be recycled to ASC by AFR reductase, a transmembrane enzyme, present in red blood cells in cooperation with other oxidoreductases and is a compensatory/protective mechanism that operates to minimize deletarious impact of oxidative stress (Lane & Lawen, 2009; Pandey & Rizvi, 2011; Rizvi, Pandey, Jha, & Maurya, 2009).

Earlier we have reported possible anti-aging and anti-diabetic role of tea catechins due to their antioxidant effect (Maurya & Rizvi, 2009; Rizvi et al., 2005). In view of reports that certain flavonoids may exert their antioxidant effect *in vivo* through activation of AFR reductase (Rizvi & Pandey, 2010), the present study was undertaken to evaluate the effect of tea catechins (EGCG, ECG, EGC, EC) on the activity of AFR reductase. The results have also been correlated with the antioxidant activity shown by tea catechins *in vitro*, measured using the ferric reducing ability/power (FRAP) and 1,1-diphenyl-2-picrylhydrazyl free radical ($DPPH^{\bullet}$) scavenging assays.

2. Material and methods

2.1. Collection of blood and isolation of red blood cells

Human venous blood from 31 healthy volunteers of both sexes (20 males and 11 females) between the ages of 21 and 30 years was

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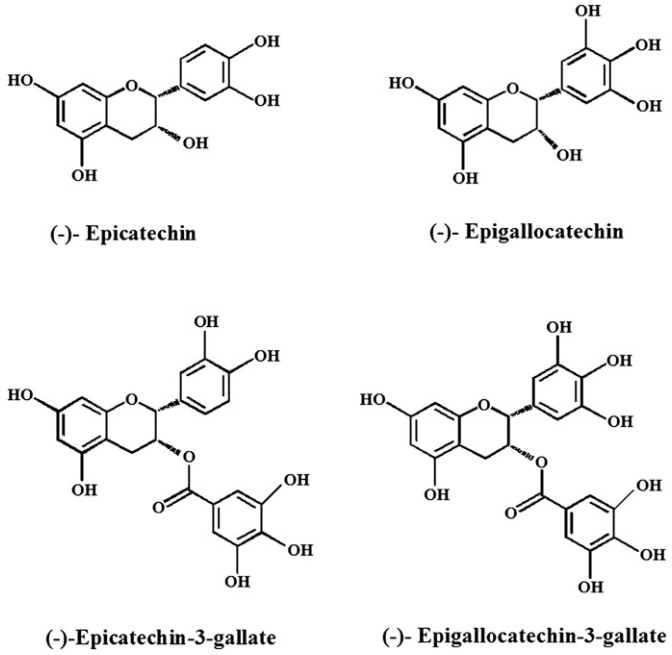

Fig. 1. Chemical structures of tea catechins.

obtained by venipuncture in heparin. The blood was centrifuged at 800×*g* for 10 min at 4 °C. The red blood cells (RBCs) were obtained after removal of plasma and buffy coat and washed twice with cold phosphate-buffered saline (PBS; 0.9% NaCl, 10 mM Na_2HPO_4, pH 7.4). All persons gave their informed consent for the use of their blood samples for the study. The protocol of study was in conformity with the guidelines of the Institutional Ethical Committee.

2.2. Incubation of human erythrocytes with catechins

Incubation of red blood cells with tea catechins was done as described previously (Rizvi & Pandey, 2010). Washed erythrocytes were suspended in 4 volumes of PBS (phosphate buffered saline) containing 5 mmol/L glucose (pH 7.4). *In vitro* effects were evaluated by incubating the erythrocytes in the presence of catechins (final concentration 1 μM and 10 μM) at 37 °C for 30 and 60 min. After this time, the suspensions were immediately centrifuged at 800×*g*, the RBCs were washed twice with at least 50 volumes of PBS and then subjected to assay for AFR reductase activity.

2.3. Determination of erythrocyte AFR reductase activity

The red cell AFR reductase activity was assayed following the method described by May, Qu, and Cobb (2004). The washed erythrocytes were hemolysed and diluted 100% (vol/vol) by addition of water followed by centrifuging for 10 min in the cold. AFR was generated in diluted hemolysates by incubating them at 37 °C in PBS (pH 7.0), containing 1 mM ascorbate, 5 unit/mL ascorbate oxidase, and 0.1 mM of nicotinamide adenine dinucleotide (NADH). The rate of NADH oxidation was measured spectrophotometrically at 340 nm for 3 min. at 37 °C. The change in NADH concentration was calculated from the slope of the resulting line, using an extinction coefficient $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$. The values were corrected in each experiment for the rate observed with lysate and reduced nucleotide alone. AFR reductase activity is reported in terms of μM NADH oxidized/min/mL of packed red blood cells (PRBC).

2.4. Measurement of antioxidant potential of catechins by FRAP assay

The ferric reducing ability/power (FRAP) of each catechins was determined by following the method of Benzie and Strain (1996). Working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 2, 4, 6-tri [2-pyridyl]-s-triazine (10 mM in 40 mM HCl) solution and $FeCl_3.6H_2O$ (20 mmol/L) solution in 10:1:1 ratio, respectively. Three milliliters of FRAP reagent was mixed with the 10 μM of each polyphenolic solution and the content was mixed vigorously. The absorbance was read at 593 nm at the interval of 30 s for 4 min. Aqueous solution of known Fe^{2+} concentration in the range of 100–1000 μmol/L was used for calibration. Using the regression equation the FRAP values (μmol Fe (II)/L) of the each polyphenolic solutions were calculated.

2.5. Determination of redical scavenging activity of tea catechins by DPPH• assay

Radical scavenging capacities of all four flavonoids were estimated by the procedure described by Miliauskas, Venskutonis, and van Beek (2004). Briefly 0.1 mL of each compound was incubated in the methanolic solution of DPPH• (0.1 mM). Absorbance at 517 nm was measured after 30 min of incubation with vigorous shaking. Methanol was used as blank reference. All the measurements are performed in triplicates. The free radical DPPH• scavenging (*i.e.* reduction) activity was calculated from the equation: Activity [% of DPPH reduction] = [(A − Ax)/A] × 100%, where A — absorbance of DPPH• solution with methanol, Ax — absorbance of a DPPH• solution with catechins.

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA. The results are reported as means ± SD.

3. Results

Incubation of tea catechins for 30 min at 5 μM final concentration significentaly activated erythrocyte AFR reductase at different rates; EGCG 31%, ECG 26%, EGC 21% and EC 14% (Fig. 3). The effect of tea catechins was dose dependent, the intensity of activation at 1 μM final concentration was less compared to that of 5 μM final concentration; EGCG 21%, ECG 16%, EGC 14% and EC 6% , however the effect of EC at 1 μM was not significant. No significant difference in the activation of AFR reductase was noted when time of incubation with tea catechins was increased from 30 min to 60 min (data not shown). All the four catechins showed ferric reducing ability as well as anti-

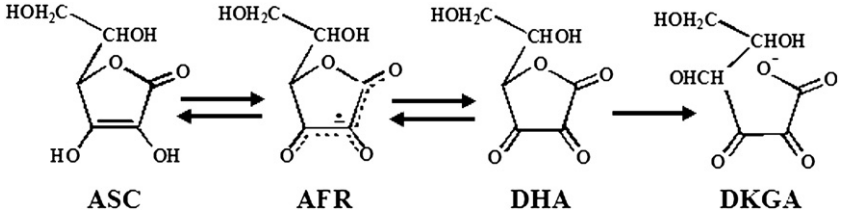

Fig. 2. Stages in ascorbate metabolism. ASC, ascorbate; AFR, ascorbate free radical; DHA, dehydroascorbate and DKGA, 2, 3-diketo-L-gulonic acid.