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Research Article

Epigallocatechin-3-Gallate Protects Erythrocyte Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase Against Oxidative Induced Damage During Aging in Humans

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Abstract

Purpose: The main purpose of this study was to investigate the protective role of epigallocatechin-3-gallate on tertiary butyl hydroperoxide induced oxidative damage in erythrocyte during aging in humans.

Methods: Human erythrocyte membrane bound Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase activities were determined as a function of human age. Protective role of epigallocatechin-3-gallate was evaluated by in vitro experiments by adding epigallocatechin-3-gallate in concentration dependent manner (final concentration range 10^{-7}M to 10^{-4}M) to the enzyme assay medium. Oxidative stress was induced in vitro by incubating washed erythrocyte ghosts with tertiary butyl hydroperoxide (10^{-5} M final concentration).

Results: We have reported concentration dependent effect of epigallocatechin-3-gallate on tertiary butyl hydroperoxide induced damage on activities of Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase during aging in humans. We have detected a significant ($p < 0.001$) decreased activity of Ca^{2+}-ATPase and Na^{+}/K^{+} -ATPase as a function of human age. Epigallocatechin-3-gallate protected ATPases against tertiary butyl hydroperoxide induced damage in concentration dependent manner during aging in humans.

Conclusion: Epigallocatechin-3-gallate is a powerful antioxidant that is capable of protecting erythrocyte Ca^{2+}-ATPase and Na^{+}/K^{+} -ATPase against oxidative stress during aging in humans. We may propose hypothesis that a high intake of catechin rich diet may provide some protection against development of aging and age related diseases.

Introduction

Tea (*Camellia sinensis*) is one of the popular beverages around the world. Tea contains polyphenolic compounds collectively known as catechins belonging to the flavonoid family. Several catechins have been identified in green tea extract,[1] but epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epicatechin (EC) have been extensively investigated. Out of all catechin, EGCG, the most abundant catechin in green tea, accounts for 65% of the total catechin content. Catechins are known to possess antioxidant,[2] anticancer,[3,4] hypoglycemic[5] and chemo-preventive[6,7] properties. Catechins are believed to react with biomolecules either directly or after cellular metabolism but the exact mechanism underlying these processes remains speculative.

Aging is the accumulation process of diverse detrimental changes in the cells and tissues with advancing age, resulting in an increase in the risks of disease and death.[8] There are many theories which attempt to explain the process of aging. The oxidative stress hypothesis offers the best valid mechanistic elucidation of the aging process and other age-related phenomenon. Aerobic cells produce ROS as a byproduct of their metabolic processes. ROS cause oxidative damage to erythrocyte membrane and biomolecules when the antioxidant defence of the body is overwhelmed. A certain amount of oxidative damage takes place even under normal conditions, however the rate of this damage increases during the aging process as the efficiency of antioxidative and repair mechanisms decreases.[9] The erythrocyte along with its membrane has always been an important medium to study aging.[10,11]

Membrane bound calcium transporting protein are important in regulating various signal functions of calcium ion (Ca^{2+}).[12] The regulation of this Ca^{2+} is performed by Ca^{2+}-ATPase. Human erythrocytes have deformability and elasticity properties which are affected by calcium ion.[13] Thus, a rise in internal Ca^{2+} leads to changes in cell shape and volume, increased cellular rigidity and hemolysis.[14,15] Such changes arise from Ca^{2+} interactions with various molecular targets. Since internal Ca^{2+} is subjected to metabolic control via Ca^{2+}-ATPase, it is expected that during aging there should be some alterations in the activity of Ca^{2+}-ATPase. The membrane bound Na^{+}/K^{+}-ATPase is the enzymatic basis of univalent cation transport.[16] Na^{+}/K^{+}-ATPase is the

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primary regulator of red blood cell (RBC) volume and hence cytoplasmic viscosity via maintaining the osmotic balance across the cell membrane.[17] It is widely believed that impairment in Na^+/K^+-ATPase activity may play a major role at the cellular level in the pathophysiology of diseases.[18]

Recently, we showed that erythrocyte Ca^{2+}-ATPase and Na^+/K^+-ATPase activity decreases during human aging.[19] We have also reported several age associated changes in human erythrocytes.[20-23] Role of tea catechin on biomarkers of oxidative stress during human aging and age related diseases has been well documented.[24-27] The present work was undertaken to evaluate concentration dependent effect of EGCG on tertiary butyl hydroperoxide (t-BHP) induced oxidative damage on erythrocyte Ca^{2+}-ATPase and Na^+/K^+-ATPase during aging in humans.

Materials and Methods

Material

Epigallocatechin-3-gallate, adenosine triphosphate (ATP) was purchased from Sigma chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA), tertiary butyl hydroperoxide, Ouabain, Imidazole was purchased from Himedia Laboratories (India). All other chemicals used were of analytical grade.

Selection of subjects

The study was carried out on normal healthy subjects of both sexes those were divided into young (18-35 years; 32 subjects), middle (36-60 years; 31 subjects) and old (> 60 years; 26 subjects) groups. The criteria for selection of subjects were the same as described earlier.[28]

Preparation of erythrocyte membrane

Human venous blood (10 ml, one time) from different healthy volunteers were obtained by venipuncture in heparin vials. The blood was centrifuged at 1800 x g for 10 min at 4°C. After collection of plasma, the buffy coat and upper 15% of the packed red blood cells, the RBC was washed twice with cold phosphate buffer saline (0.9% NaCl, 10 mM Na_2HPO_4, pH 7.4). The erythrocyte membrane from leukocyte free red cells was prepared following the method of Marchesi and Palade.[29]

Determination of Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity was assayed as described earlier.[19] Briefly, 2.25 ml of the assay mixture contained 80 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 18 mM Tris-HCl (pH 7.4), 0.1 mM ouabain, 0.1 mM EGTA, 0.2 ml of the membrane containing 0.4 to 1.5 mg protein per ml and ± 0.2mM $CaCl_2$. The reaction was initiated by the addition of 0.1 ml of 30 mM ATP. After 30 min at 37°C, the reaction was stopped by adding 3.5 ml of a solution containing 0.5 M H_2SO_4, 0.5% ammonium molybdate and 2% SDS. The amount of liberated inorganic phosphate was estimated.[30] The Ca^{2+}-ATPase activity is expressed in terms of micro mole of Pi (inorganic phosphate) released / hr / mg membrane protein at 37°C.

Determination of Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity was measured as described earlier.[19] The final assay mixture contained 0.4 to 0.9 mg membrane protein per ml, 140 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 30 mM imidazole (pH 7.25), ± 5 x 10^{-4} M ouabain and 6 mM ATP. Incubation was carried out for 30 min at 37°C; the reaction was stopped by adding 3.5 ml of a solution containing 0.5 M H_2SO_4, 0.5% ammonium molybdate and 2% SDS. The amount of liberated inorganic phosphate was estimated.[30] The Na^+/K^+-ATPase activity has been expressed in terms of micro mole of Pi released / hr / mg membrane protein at 37°C.

Protein determination

Erythrocyte membrane protein was estimated by following the method of Lowry.[31]

In vitro experiments with EGCG and induction of oxidative stress

The effect of EGCG on erythrocyte membrane Ca^{2+}-ATPase and Na^+/K^+-ATPase were studied as described earlier.[27] In brief, in vitro experiments were carried out by adding EGCG in concentration dependent manner (final concentration range: 10^{-7}M to 10^{-4}M) to the enzyme assay medium and incubating at 37°C for 60 minutes prior to enzyme assay. In parallel control experiments, the assay medium was incubated without EGCG. Oxidative stress was induced in vitro by incubating washed erythrocyte ghosts with t-BHP (10^{-5} M final concentration).

Statistical analysis

Statistical analysis was carried out using Graph pad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. Data have been presented as means ± S.D. $p < 0.05$ is considered statistically significant.

Results and Discussion

We have reported concentration dependent effect of EGCG in young, middle and old groups on t-BHP induced oxidative damage on erythrocyte Ca^{2+}-ATPase and Na^+/K^+-ATPase activities. The use of different concentrations of EGCG in our experiments were because, the range represent the average plasma EGCG level in regular green tea drinks.[32] Any change or damage to erythrocyte membranes bound enzymes will be resulted in altered activity.

As shown in Figure 1 (a, b & c), the membranes were exposed to t-BHP (10^{-5}M final concentration) with and without EGCG. The t-BHP significantly ($p < 0.05$) inhibited erythrocyte membrane bound Ca^{2+}-ATPase activity in different age groups (control vs t-BHP) viz. young (0.52 ± 0.032 vs 0.33 ± 0.021) (Figure 1a); middle (0.32 ± 0.026 vs 0.22 ± 0.021) (Figure 1b) and old (0.21± 0.026 vs 0.11 ± 0.021) (Figure 1c). The concentration dependent effect of EGCG (10^{-7}M to 10^{-4}M) in different age groups have been reported as fallows: young (0.42 ±

0.012; 0.44 ± 0.025; 0.48 ± 0.033; 0.52 ± 0.023 respectively**)** , middle (0.24 ± 0.022; 0.32 ± 0.015; 0.36 ± 0.033; 0.37 ± 0.033 respectively) and old (0.16 ± 0.022; 0.22 ± 0.015; 0.24 ± 0.033; 0.24 ± 0.033 respectively).

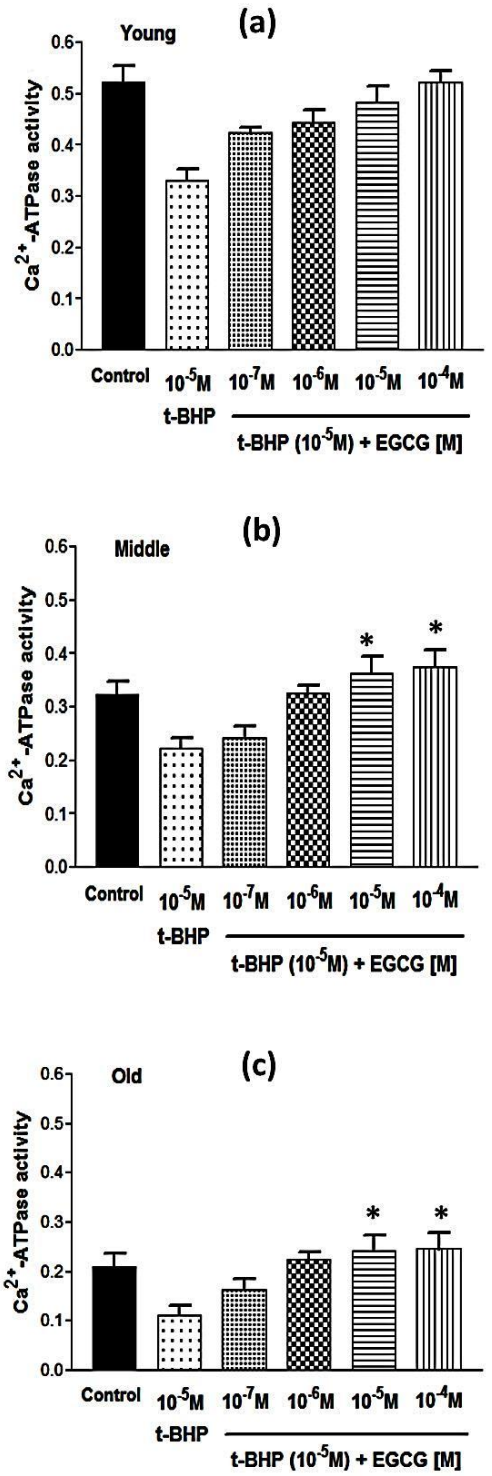

Figure 1. Concentration dependent effect of EGCG on t-BHP induced oxidative damage on erythrocyte Ca^{2+}-ATPase activity. Fig.1a-Young, Fig. 1b-Middle and Fig.1c-Old. Enzyme activity is expressed in terms of micro mole of Pi released / hr / mg membrane protein at 37°C. Values are means ± S.D. * p<0.05 as compared to control.

Subjecting erythrocytes membrane to increased oxidative stress by incubating with t-BHP causes significant ($p < 0.05$) decreased activity of Na^+/K^+ - ATPase as a function of human age. Control vs t-BHP values have been shown in young (0.043 ± 0.004 vs 0.031 ± 0.003) (Figure 2a), middle (0.038 ± 0.003 vs 0.028 ± 0.002) (Figure 2b) and old (0.021 ± 0.002 vs 0.012 ± 0.001) (Figure 2c). The presence of EGCG in the incubation medium protect erythrocyte membrane from t-BHP induced oxidative damage in age dependent manner, as evidenced by increase in Na^+/K^+ - ATPase activities in all age groups. Figure 2 (a, b & c) shows concentration dependent effect of EGCG (10^{-7}M to 10^{-4}M) in young (0.040 ± 0.001; 0.047 ± 0.004; 0.049 ± 0.001; 0.050 ± 0.001 respectively**)**, middle (0.032± 0.002; 0.042 ± 0.004; 0.044 ± 0.001; 0.046 ± 0.001 respectively) and old (0.012 ± 0.001; 0.020 ± 0.002; 0.024 ± 0.003; 0.026 ± 0.002 respectively).

It has been shown that in vitro concentration dependent effect of EGCG on t-BHP induced oxidative damage, which results in significant increase in the activities of Ca^{2+}-ATPase and Na^+/K^+-ATPase. The effect was more pronounced in old age group. At high concentration of EGCG, the activities of Ca^{2+}-ATPase and Na^+/K^+-ATPase reaches normal value and even higher in middle and old age groups, while at low concentration the activity is not found to be altered significantly as a function of age. The results are in agreement with previous reports[33] that, EGCG can interact with peroxyl radicals and inhibit lipid peroxidation.[26] Results are supported by our previous studies that lipid peroxidation increases while antioxidant level decreases during aging in humans.[34,35] It has been shown that these membrane bound enzymes are very susceptible to oxidative damage.[36] Taking all above results into the consideration, we may propose that t-BHP inhibited ATPase activities, either by interacting with ATPase directly or by interacting with lipids in the membrane indirectly during aging in humans. Decrease in the activity of ATPases were either due to direct oxidation of protein or lipid component of these enzymes, or due to oxidation of membrane lipids, which could affect membrane fluidity leading to inhibition of Ca^{2+}-ATPase and Na^+/K^+-ATPase activities. The protective effect of EGCG was either due to scavenging peroxides or due to blocking the oxidation of membrane lipids and proteins but the exact mechanism underlying these processes remains speculative.

Conclusion

In conclusion, EGCG could protect human erythrocyte membrane against oxidative damage during aging in humans and act as a powerful antioxidant. The normal human body has very complex and efficient antioxidant system consisting with number of interrelated antioxidant compounds and enzymes. Mechanism(s) that are thought to be involved in the increased oxidative stress as a function of human age include not only ROS generation but also change in the tissue / plasma content

and the activity of antioxidant defense system. We can suggest that high intake of catechin rich diet by higher age groups may provide some protection against development of age related diseases and may slow down aging process.

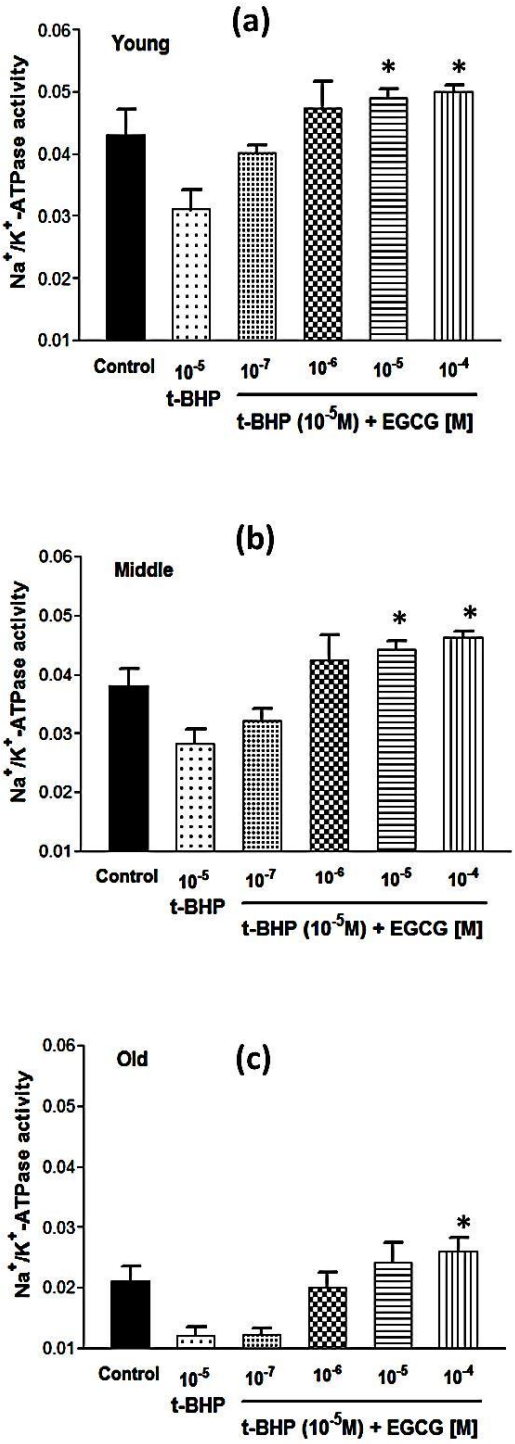

Figure 2. Concentration dependent effect of EGCG on t-BHP induced oxidative damage on erythrocyte Na^+/K^+-ATPase activity. Fig. 2a-Young, Fig. 2b-Middle and Fig. 2c-Old. Enzyme activity is expressed in terms of micro mole of Pi released / hr / mg membrane protein at 37°C. Values are means ± S.D. * $p<0.05$ as compared to control.

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Ethical issues

The protocol of study was in conformity with the guidelines of the Institutional Ethical Committee.

Conflict of interest

The authors report no conflicts of interest.

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