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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Protection of protein carbonyl formation by quercetin in erythrocytes subjected to oxidative stress

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Abstract Quercetin, 3,3',4',5,7-pentahydroxyflavone, is one of the most abundant naturally occurring polyphenolic compounds. Evidences suggest that quercetin has biological properties that may play an important role in prevention of human diseases, such as cancer, cardiovascular diseases, diabetes, and allergies. Many of the biological actions of this flavonoid have been attributed to its antioxidant properties. In the present study, we have determined the protection of protein carbonyl formation by quercetin in erythrocytes subjected to oxidative stress. In vitro oxidative stress in human erythrocytes was induced by incubating with 10^{-5} M tert-butylhydroperoxide. This resulted in a significantly increased level of carbonyl content in erythrocyte membrane. Treatment with quercetin caused a decrease in the carbonyl content. The effect of quercetin was concentration and time-dependent. The protection of carbonyl formation in proteins substantiates the strong biological antioxidant property of quercetin.

Keywords Erythrocyte · Oxidative stress · Quercetin · Carbonyl

Introduction

Quercetin, 3,3',4',5,7-pentahydroxyflavone, is one of the most abundant naturally occurring polyphenolic compounds, widely distributed as secondary metabolites in the plants (Fig. 1). Belonging to the flavonoid group of polyphenols, quercetin is ubiquitously present in foods, including vegetables, especially onions (*Allium cepa*), fruits, tea, and wine (Boots *et al.*, 2008).

Reactive oxygen species (ROS) are continuously produced during cell metabolism. Under normal conditions, they are scavenged and converted to nonreactive

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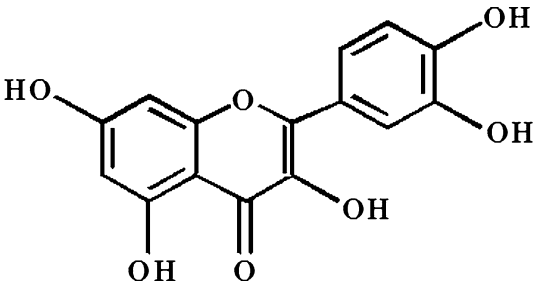

Fig. 1 Chemical structure of quercetin

species by different intracellular, enzymatic, and nonenzymatic antioxidant systems (Hyman *et al.*, 2005). Overproduction or an ineffective elimination of ROS may induce oxidative stress and cause damage to all types of biomolecules, such as proteins, lipids, and nucleic acids (Droge, 2002). A certain amount of oxidative damage takes place even under normal conditions; however, the rate of this damage increases during aging and other pathological events, as the efficiency of antioxidative and repair mechanisms decreases, leading to the condition of oxidative stress (Gil *et al.*, 2006).

The attack by ROS against proteins modifies amino acid (lysine, arginine, proline, and histidine) residues generating carbonyl moieties, which has been identified as an early marker for protein oxidation and is used as a measure of protein damage (Levine *et al.*, 1990).

There is overwhelming evidence to suggest that nutritional sources of antioxidants, such as fruits, vegetables, tea, or wine would attenuate tissue damage caused by oxidative challenges (Cao *et al.*, 1998). Polyphenolic compounds abundantly present in these nutritional sources could play a major role in enhancing the antioxidant system (Hollman *et al.*, 1996; Rizvi *et al.*, 2005).

Several in vivo and in vitro studies have been conducted to evaluate the biological effects of quercetin. Evidence suggests that quercetin has biological properties that may play an important role in the prevention of human diseases, such as cancer, cardiovascular diseases, diabetes, and allergies (Liu *et al.*, 2008; Reutrakul *et al.*, 2007). Many of the biological actions of this flavonoid have been attributed to its antioxidant properties (Perez-Vizcaino *et al.*, 2006; Reutrakul *et al.*, 2007). We report the protective effect of quercetin on carbonyl formation in erythrocytes subjected to oxidative stress by incubating with tert-butylhydroperoxide (t-BHP).

Materials and methods

Erythrocyte ghost preparation

Human venous blood from different healthy volunteers was obtained by venipuncture in heparin. The blood was centrifuged at 1,800 *g* for 10 minutes at 4°C. After the removal of plasma, buffy coat, and upper 15% of the packed red blood cells (RBCs), the RBCs were washed twice with cold PBS (0.9% NaCl, 10 mM

Na_2HPO_4, pH 7.4). Erythrocyte ghosts from leucocyte-free RBCs were prepared by osmotic shock procedure by the methods of Marchesi and Palade (1967). The protocol of study was in conformity with the guidelines of the Institutional Ethical Committee.

Determination of membrane protein carbonyls

Erythrocyte membrane protein carbonyls were measured according to procedure of Levine *et al.* (1990): 0.2 ml of erythrocyte membrane samples in PBS were taken in two tubes as test and control, 4.0 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) prepared in 2 M HCl was added to the test sample, and 4.0 ml of 2 M HCl alone was added to the control sample. The contents were mixed thoroughly and incubated for 1 hour in the dark at 37°C. The tubes were shaken intermittently every 10 minutes to facilitate the reactions with proteins. After that, 20% TCA (w/v) was added to both tubes and the mixture left in ice for 10 minutes. The tubes were then centrifuged at 3,500 rpm for 20 minutes to obtain the protein pellets. The supernatant was carefully aspirated and discarded. The protein pellets were washed three times with ethanol: ethyl acetate (1:1, v/v) solution to remove unreacted DNPH and lipid remnants. Finally protein pellets were dissolved in 6 M guanidine hydrochloride and incubated for 10 minutes at 37°C. The insoluble materials were removed by centrifugation. Carbonyl content was determined by taking the spectra of the supernatant at 370 nm. Each sample was read against the control. The carbonyl content was calculated by using an absorption coefficient (e) of 22,000 M^{-1} cm^{-1}, and data were expressed in nmol/mg protein. The erythrocyte membrane protein content was determined by the method of Lowry *et al.* (1951), using BSA as standard.

Induction of oxidative stress

Oxidative stress was induced in vitro by incubating washed erythrocyte ghosts with 10^{-5} M tert-butylhydroperoxide (t-BHP) with or without quercetin. The concentration of t-BHP used in the present study to induce oxidative stress in erythrocytes was in the range of concentrations used in other previously published reports (Luqman and Rizvi, 2006).

In vitro experiments with quercetin

Erythrocyte ghosts (0.8–1.5 mg of protein) were incubated with the quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) at different doses in PBS (pH 7.4) for 1 hour at 37°C before the estimation of protein carbonyls formation. Parallel control experiments also were performed in which quercetin was replaced with an equal amount of solvent.

Statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Results showing $p < 0.05$ were assumed to be significant.

Results and discussion

Epidemiological studies, using data from consumption of quercetin-rich fruits and vegetables, advocate the beneficial health effects of quercetin. These effects include reduced rate of coronary heart diseases, cancer, incident of asthma, infections, and inflammation (Reutrakul *et al.*, 2007). The possible mechanism by which quercetin exerts these beneficial effects is thought to be its antioxidant activity (Liu *et al.*, 2008).

Subjecting erythrocytes to oxidative stress (in vitro) by incubating them with t-BHP (10^{-5} M) caused an approximately 250% increase in the protein carbonyl group content above the basal value. Incubation of erythrocytes with quercetin showed a significant protection against the t-BHP-induced oxidative stress as evidenced by the decrease in the protein carbonyl group content. We observed that the effect of quercetin is dose/concentration-dependent; the protective effect increases with an increase in the concentration of quercetin from 0.1 μM to 100 μM (Fig. 2).

Erythrocytes are highly susceptible to the oxidative damage due to the high cellular concentration of oxygen and hemoglobin—a potentially powerful promoter for the oxidative processes (Bryszewska *et al.*, 1995). Oxidative modification of proteins may be selective and specific. Use of protein carbonyls as index of oxidative stress has some advantages compared with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins (Dalle-Donne *et al.*, 2003). Accumulation of protein carbonyls is associated with a number of diseases, including amyotrophic lateral

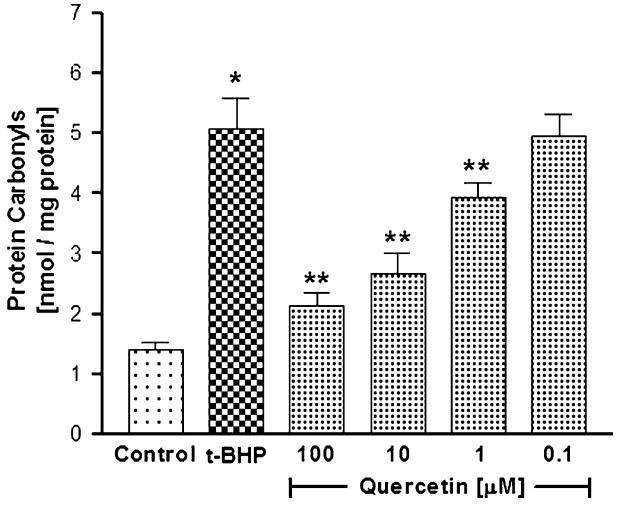

Fig. 2 Dose-dependent effect of quercetin on protein carbonyl content in oxidatively stressed human erythrocytes. *Incubation with t-BHP caused increase in protein carbonyl group level ($p < 0.001$) compared with control. **Treatment with quercetin shows significant protection against t-BHP induced stress at different concentration at 100 μM ($p < 0.001$), 10 μM ($p < 0.01$), and 1 μM ($p < 0.05$). Effect at concentration 0.1 μM was not significant. Carbonyl content is expressed as nmol/mg protein

sclerosis, Alzheimer's disease, respiratory distress syndrome, muscular dystrophy, and rheumatoid arthritis (Berlett and Stadtman, 1997).

Our results show that quercetin can protect erythrocytes from oxidative stress under in vitro conditions. The same conditions are thought to occur in vivo, and we hypothesize that quercetin may provide protection against oxidation induced damage to membrane proteins under conditions that challenge the body's redox status. Protection of carbonyl formation in t-BHP induced oxidative stressed erythrocytes by quercetin in micromolar concentrations assumes significance because it has been reported that plasma quercetin levels after the intake of a quercetin-rich diet are usually in micromolar range $\approx$ 1–2 μM (Conquer *et al.*, 1998; Wiczkowski *et al.*, 2008). It has been reported that quercetin is significantly absorbed and its peak plasma concentration was achieved after 2–2.7 hours of administration (McAnlis *et al.*, 1999).

The dose-dependent effect of quercetin can be explained by previously reported observations in which it has been documented that the repeated quercetin supplementation could attain a considerable plasma level, sometimes reaching up to 4 μmol; a higher plasma concentration is associated with enhanced activity (Manach *et al.*, 2005; Wiczkowski *et al.*, 2008).

Quercetin is considered the most potent scavenger of ROS among the other members of the flavonoid family. The antioxidative capability of quercetin is attributed to the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for free radical scavenging (Fig. 1) (Boots *et al.*, 2008). It also is suggested that the quercetin substantially empowers the endogenous antioxidant shield due to its contribution to the total plasma antioxidant capacity, which is six times higher than trolox, an antioxidant generally used as a reference (Arts *et al.*, 2004).

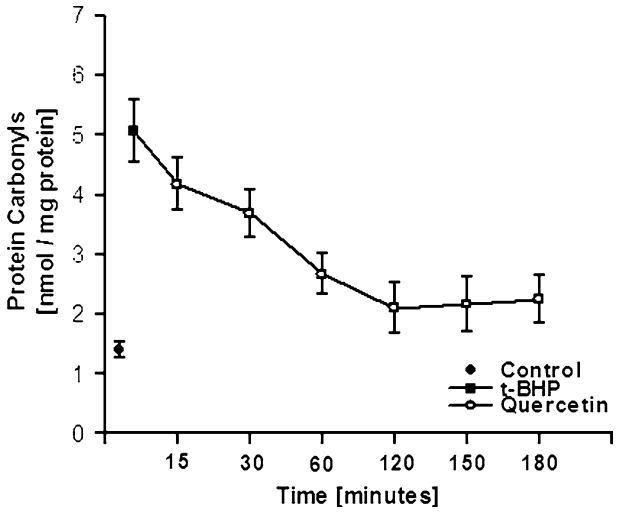

Fig. 3 Time-dependent effect of quercetin on protein carbonyl content in oxidatively stressed erythrocytes. Carbonyl content is expressed as nmol/mg protein

In our experiments, we also evaluated the time-dependent effect of quercetin. A significant protective effect against protein oxidation was observed after only 15 minutes of incubation with quercetin. The protective effect increases gradually up to 120 minutes, after which a slight reduction is observed (Fig. 3).

In view of reports of the protective effect of quercetin on oxidation induced alterations in erythrocyte malondialdehyde, reduced glutathione, and membrane–SH groups (Coskun *et al.*, 2005; Rizvi and Mishra, 2009), our present findings on the protection of carbonyl formation in proteins substantiate the strong biological antioxidant property of quercetin. We hypothesize that quercetin-rich diet may reduce the damage of biomolecules in several degenerating diseases, including cardiovascular, cancer, and diabetes.

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