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FLAVONOID TREATMENT OF HUMAN ERYTHROCYTES PROTECTS MEMBRANE-BOUND NADH-METHEMOGLOBIN REDUCTASE AGAINST OXIDATIVE DAMAGE

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The activity of NADH-methemoglobin reductase in membranes isolated from human erythrocytes subjected to the action of phenylhydrazine in sublytic concentration was studied. A decrease of enzyme activity was shown dependent on the concentration of phenylhydrazine. After exposure of human erythrocytes to 1.0–1.5 mM phenylhydrazine (15 min, 37°C) activity of the membrane-bound NADH-methemoglobin reductase was about 40% of the initial level. Increased level of membrane-bound methemoglobin was also observed. It was found that preincubation of human erythrocytes with 1×10^{-5} M quercetin or 1×10^{-5} M genistein-C-glucoside in isotonic Na-phosphate buffer, pH 7.4 (2 hours, 37°C) prevented the phenylhydrazine-induced inhibition of activity membrane-bound NADH-methemoglobine reductase. The observed effects of two flavonoids studied show their significant potential as antioxidant (radical-scavenging) agents for protection of cells from free radicals.

INTRODUCTION

Many xenobiotics of diverse structure may induce oxidative stress in erythrocytes. Erythrocytes have a powerful antioxidant protection system. However, under conditions of high concentration of free radicals in erythrocytes or insufficiency of the primary antioxidant protection the oxidative damage of the erythrocyte membrane components leads to loss of the ability of erythrocytes to transfer O_2 and CO_2, and brings about cell hemolysis. Oxidant-induced hemolysis can also take place during some hematological diseases, during physiological aging of erythrocytes, and in some other cases (Petty, Zhou & Zheng, 1991; Edwards & Fuller, 1996; Slobozhanina, Kozarezova & Klimkovich, 1998).

In erythrocytes under oxidative stress there is a considerable rise in the level of methemoglobin, which is known to be incapable of reversible oxygen binding. Under physiological conditions, human erythrocytes contain ~1–2% methemoglobin. For converting methemoglobin into oxyhemoglobin there are two enzymatic systems in erythrocytes, one of which is related to glycolysis and the other is associated with the pentose phosphate pathway. Correspondingly, two types of enzymes function in erythrocytes: NADH-methemoglobin reductase and NADPH-methemoglobin reductase, both having cytoplasmic and membrane-bound forms. It is generally assumed that the reduction of oxidized hemoglobin with the participation of membrane-bound NADH-methemoglobin reductase is of greatest physiological significance. Since it is known that hemoglobin actively reacts with oxidants and that its oxidation products can cause stress, one can suppose that the persistence of the activity of the reductases that reduce oxidized hemoglobin plays an important role in maintaining the viability of erythrocytes.

In our previous paper we showed that phenylhydrazine-induced oxidative stress decreased the activity of membrane-bound NADH-methemoglobin reductase in human erythrocytes and increased the amount of membrane-bound methemoglobin (Slobozhanina, Lukyanenko & Kozlova, 2000).

The purpose of the present work was to define antioxidant activity of bioflavonoids in phenylhydrazine-induced oxidative stress in human erythrocytes.

MATERIALS AND METHODS

Experiments were carried out on erythrocytes from the donor blood provided by the Hematology and Blood Transfusion Research Institute, Ministry of

Health, Belarus. Erythrocytes were separated by centrifugation at 3000 g and 4°C, and washed thrice with 5 mM sodium phosphate buffer containing 150 mM NaCl (buffer A). Control erythrocytes and cells preincubated with quercetin or genistein-C-glucoside were exposed to 1 mM phenylhydrazine hydrochloride at 37°C in buffer A for 15 min and then washed three time in buffer A.

Erythrocyte membranes (ghosts) were isolated by the method of Dodge, Mitchell and Hanahan (1963). Protein concentration in erythrocyte ghosts was estimeted by the Lowry micromethod (Markwell, Haas & Tolbert, 1978). The membrane-bound NADH-methemoglobin reductase activity was determined at 25°C according to Papandreou and Rakitzis (1989). The oxyhemoglobin and methemoglobin content was determined spectrophotometrically (Stus & Rozanova, 1992).

The following reagents were used: phenylhydrazine hydrochloride (Sigma, USA); quercetin (Chemapol, Czechoslovakia); $NADH_2$ (Reanal, Hungary); potassium ferricyanide, NaH_2PO_4, Na_2HPO_4, and NaCl (all of reagent grade or special purity grade, Reakhim, CIS). Genistein-C-glucoside extracted from buds of *Lupinus luteus* with 96% ethanol, were further purified by high-performance liquid chromatography.

The experimental results were statistically assessed by the Student's "t" test.

RESULTS

The experiments showed that in membranes isolated from human erythrocytes after 15-min-long incubation in a medium containing phenylhydrazine hydrochloride at sublytic concentrations, the NADH-methemoglobin reductase activity was much lower than that in the reference sample decreasing by 10–15% the phenylhydrazine concentration 0.25–0.5 mM and by 70% at concentration 1.5 mM (Fig. 1). Increased level of membrane-bound methemoglobin was also observed. Our experiments demonstrated that a 2-hour-long incubation of erythrocytes in buffer A containing 1×10^{-5} M quercetin or 1×10^{-5} M genistein-C-glucoside from *Lupinus luteus* does not change the activity of NADH-methemoglobin reductase (Fig. 2). A loss (about by 40%) of flavonoids from the medium, in which of erythrocytes were incubated, was observed spectrophotometricaly which indicates absorption of these substances by the cells.

It was found that preincubation of human erythrocytes in buffer A with 1×10^{-5} M quercetin and 1×10^{-5} M genistein-C-glucoside prevented phenylhydrazine-induced inhibition of activity NADH-methemoglobin reductase (Fig. 3). In earlier work

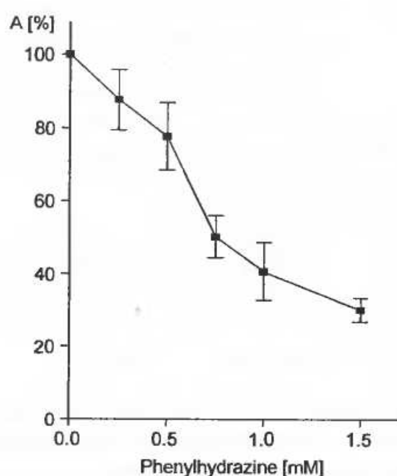

Fig. 1. Effect of phenylhydrazine on the activity of NADH-methemoglobin reductase in membranes extracted from erythrocytes incubated in media at different phenylhydrazine hydrochloride concentrations for 15 min at 37°C and then washed twice in an isotonic buffered NaCl solution at pH 7.4. The membrane-bound NADH-methemoglobin reductase activity in reference samples was taken as 100%. Data represent means ± SD, n = 6

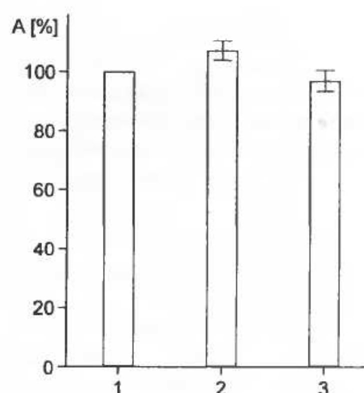

Fig. 2. Effect of flavonoids on the activity of membrane-bound NADH-methemoglobin reductase in human erythrocytes. Cells was preincubated at 37°C for 2 hr in: 1 – Buffer A (control); 2 – Buffer A + 1×10^{-5} M quercetin; 3 – Buffer A + 1×10^{-5} M genistein-C-glucoside. Membranes were extracted from erythrocytes after incubation of the cells with flavonoids. The NADH-methemoglobin reductase activity in control samples was taken as 100%. Data represent means ± SD, n = 3

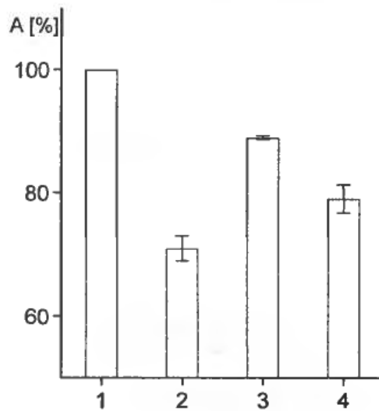

Fig. 3. Effect of preincubation of human erythrocytes with flavonoids on the activity of membrane-bound NADH-methemoglobin reductase after treatment with of 1 mM phenylhydrazine hydrochloride. Cells was incubated in: 1 – Buffer A (control); 2 – Buffer A + 1 mM phenylhydrazine hydrochloride; 3 – Buffer A + 1 × 10^{-5} M quercetin + 1 mM phenylhydrazine hydrochloride; 4 – Buffer A + 1 × 10^{-5} M genistein-C-glucoside + 1 mM phenylhydrazine hydrochloride. Data represent means ± SD, n = 4

similar antioxidative effects of flavonoids on human erythrocytes has been found (Slobozhanina & Laman, 1999). It is known from the literature that the reaction of phenylhydrazine with hemoglobin gives rise to free radicals (Goldberg & Stern, 1977). It can be assumed that in our experiments quercetin and genistein-C-glucoside from *Lupinus luteus* manifest radical-scavenging properties.

The obtained results demonstrate that quercetin and genistein-C-glucoside show antioxidative effect against damage of erythrocyte by phenylhydrazine.

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