Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2005, 149(2):497–500.

THE EFFECT OF QUERCETIN AND GALANGIN ON GLUTATHIONE REDUCTASE

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Received: June 10, 2005; Accepted: September 25, 2005

Key words: Flavonoids/ Glutathione reductase/ Diaphorase activity.

Quercetin and galangin can change the activity of glutathione reductase. Quercetin (a catechol structure in the B-ring) and galangin (any hydroxyl group in the B-ring) have different biological activities but, both possess high antioxidant abilities. Quercetin during the antioxidative action, is converted into an oxidized products (*o*-semiquinone and *o*-quinone), and subsequently glutathionyl adducts may be formed or SH-enzyme can be inhibited. We have tried to see whether inhibition of glutathione reductase (GR) can be influenced by preincubation of enzyme with NADPH (a creation of reduced form of enzyme, GRH_2) and whether diaphorase activity of the enzyme is decreased by these flavonoids. The results confirmed that quercetin inhibits GRH_2 and inhibition is reduced by addition of EDTA or *N*-acetylcysteine. Both of flavonoids have no effect on diaphorase activity of glutathione reductase and this enzyme could increase the production of free radicals by catalysis of reduction of *o*-quinone during action of quercetin *in vivo*.

INTRODUCTION

Flavonoids are frequently components of human diet and multiple mechanisms have been identified for their biological and pharmacological effects[1–3]. The major effects of flavonoids may be the result of radical scavenging. Another possible mechanism by which flavonoids act is through interaction with various enzyme systems. Their cellular activities are ambiguous, with some activities being mutually opposing, for example, flavonoids protect cells from "oxidative stress" however pro-oxidant properties of these compounds could contribute to tumor cell apoptosis or genotoxicity[4–6]. The intracellular ratio GSH/GSSG is an important indicator of the cellular redox state[7]. Flavonoids can modify redox state of glutathione, GS-flavonoid conjugate or oxidation of GSH to GSSG can arise from reactions of GSH with *o*-semiquinone[8]. In addition the effects of flavonoids on the glutathione-related enzymes have been demonstrated[9–12]. Glutathione reductase (EC 1.6.4.2, GR) has a prominent role because the product of GSH oxidation, GSSG, is rapidly converted back to GSH by this enzyme. Flavonoids have been described to inhibit glutathione reductase isolated from different sources[9–12]. Elliott et al.[9] shown that the most potent inhibitors of yeast glutathione reductase are luteolin and quercetin and they estimated the IC_{50} values (170 μM, resp. 280 μM).

Recently we have studied the modulation of cis-Pt induced apoptosis by quercetin and galangin. Flavonoids (at low concentrations) differentially modulated cis-Pt-induced apoptosis and the involvenment of intracellular glutathione was observed[13,14]. Our work hypothesis is that one of the reason of the diverse action of quercetin and galangin is related with uneven effect of flavonoids on the glutathione reductase.

In this paper we have investigated the influence of these flavonoids at low concentration (from 25 to 100 μM) on the activities of the glutathione reductase from yeast, GSSG-reductase activity and diaphorase activity of GR has been examined. Yeast GR and human GR are similar in topology[15,16], thus allowing the use the yeast enzyme as a model for the inhibition studies.

MATERIAL AND METHODS

Chemicals

Glutathione reductase from baker's yeast, NADPH, reduced and oxidised forms of glutathione (GSH and GSSG), *N*-acetylcysteine (NAC) and flavonoids (quercetin (QU), galangin (GA)) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Flavonoids were dissolved in dimethyl sulphoxide (DMSO, Sigma). The stock solutions of flavonoids (0.1 M) were stored at –20 °C. EDTA, 2,6-Dichlorophenolindophenol (DPIP) and all other chemicals were obtained from Lachema Brno (Czech Republic).

Enzyme assays

Glutathione reductase activity was measured by monitoring the oxidation of NADPH at 340 nm at 25 °C for 3 min (PU 8750 UV/VIS Scanning Spectrophotometer PHILIPS). The standard GR reaction medium contained 100 mM phosphate buffer, pH 7.4, 5–100 μM NADPH, 1 mM GSSG and 20 μl of dilute GR. Enzyme suspension (198 U/mg prot) was diluted 200-fold with the 50 mM

phosphate buffer and kept at 4 °C. Total volume was 2 ml and the reaction was started by adding NADPH or GSSG, the initial velocity of GR reaction was measured by the slope of recorded tracing. Glutathione reductase activity was expressed as nmole NADPH oxidised/min/mg protein, using $\varepsilon = 6.22\ mM^{-1}.cm^{-1}$.

GR-diaphorase activity was measured by the rate of DPIP reduction at 600 nm ($\varepsilon = 19.0\ mM^{-1}cm^{-1}$) using NADPH as electron donor. The reaction medium contained 0.1 M phosphate buffer, pH 7.4, 0.2 mM NADPH and GR. The action was started adding DPIP (40 μM). DT-diaphorase activity was expressed as nmol DPIP reduced/min/mg protein.

All data points in the figure and tables represent the mean of three or more experiments ±SD.

RESULTS AND DISCUSSION

Glutathione reductase is ubiquitous FAD-containing enzyme. It catalyses the reaction

$$GR + NADPH \rightleftharpoons GRH_2 + NADP$$
$$GRH_2 + GSSG \rightleftharpoons GR + 2GSH,$$

where the intermediate GRH_2 is the stable 2-electron reduced form of the enzyme. NADPH reacting first with catalytic site of the enzyme to produce the GRH_2 which in turn binds and reduces GSSG (bi-bi ping-pong mechanism)[16]. The enzyme has diaphorase activity except GSSG-reductase activity. Enzyme is inactivated by SH-reagents, e.g. *N*-ethylmaleimide strongly influenced GSSG-reduction but the effect on diaphorase activity is very slight[17, 18]. If only quercetin could modify enzyme at the intermadiate state – GRH_2 than preincubation of GR with QU increases its inhibition effect and contrary inhibition efficiency of galangin could not be dependent on preincubation of enzyme with NADPH.

At first the effect of quercetin and galangin on GSSG-reductase activity has been estimated at low concentration (25 μM). Flavonoids were added to the enzyme whithout or after preincubation of enzyme with NADPH (1 min). The inhibition effect of galangin was weak and did not depend on preincubation with the coenzyme (Table 1).

Table 1. The effects of flavonoids at 25 μM concentration on GR activity without and after 1 min preincubation with NADPH

	Remaining activity [%]	
	preincubation	without preincubation
control	100	100
Quercetin	41.8±0.8	78.3±0.9
Galangin	65.0±0.7	68.6±0.9

Remaining activity of GR was measured as described in "Materials and Methods", 100 μM NADPH and 1 mM GSSG were used. GR activity of control: 24.62±0.12 μmol NADPH/min/mg protein.

As seen in Table 1, quercetin is a potent inhibitor but its effect without preincubation with NADPH was obscure. GSSG-reductase activity decreased about 60% only after preincubation of GR with NADPH.

The kinetic studies showed that quercetin inhibition could be an acompetetive with respect to NADPH but only at low concentration of NADPH (< 35 μM) (Fig. 1 inside). The inhibition effect was considerably higher when NADPH was kept at higher concentration (≥ 50 μM) (Fig. 1).

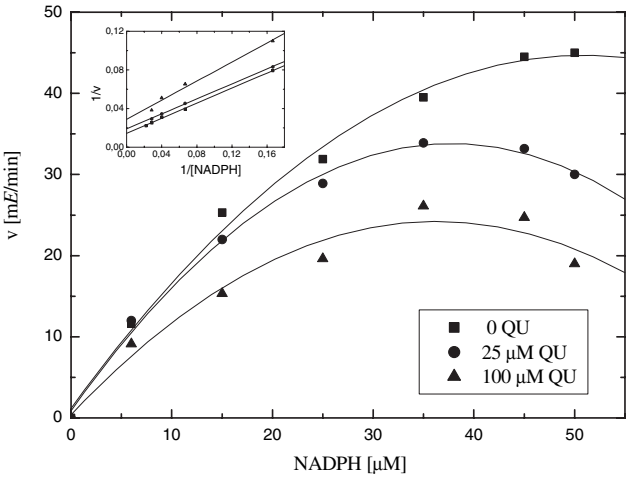

Fig. 1. The effect of quercetin on enzyme kinetic plots. Enzyme activity was measured as described in "Materials and Methods" at different NADPH concentration and at 1 mM GSSG. Inset: Lineweaver-Burk plot. Values of y-axis are absorbance increments at 340 nm for 1 min and reciprocal of absorbance increments (Lineweaver-Burk plot).

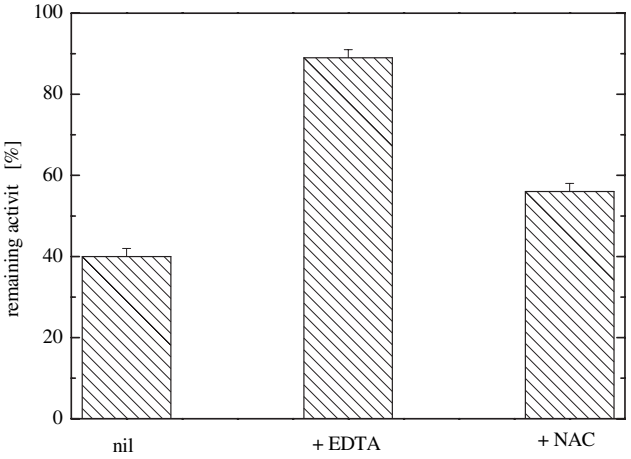

Fig. 2. Effects of EDTA and NAC on quercetin inhibition of glutathione reductase. The enzyme activity was measured as described in "Materials and Methods", 100 μM NADPH and 1 mM GSSG were used. 25 μM QU was added after 1 min preincubation of the enzyme with NADPH and 200 μM NAC or 200 μM EDTA. GR activity of control: 24.42±0.22 μmol NADPH/min/mg protein.

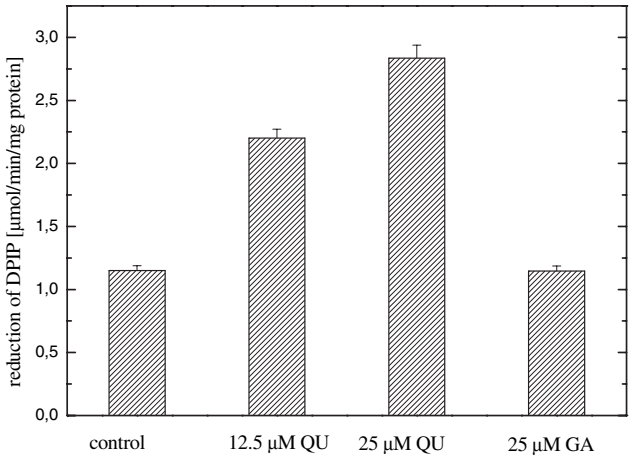

Fig. 3. Effects of quercetin and galangin on diaphorase activity of glutathione reductase. Diaphorase activity was measured as described in "Materials and Methods".

Two possible modes of quercetin action could be considered, formation of ROS in the presence of catalytical amounts of metal ions and irreversible modification of the enzyme by the oxidized products from this catechol-containing flavonoid. Transition metal ions are known to catalyze the oxidation of quinols[19]. A quite similar phenomenon has been demonstrated for the interaction of rabbit reticulocyte 15-lipoxygenase-1 with flavonoids[20].

As seen in Fig. 2, the elimination of trace of metal ions by the addition of 200 μM EDTA protected GR against inhibition by 25 μM quercetin. When a *N*-acetylcysteine (200 μM) was used for a protection of GR, the enzyme activity was protected only partially (Fig. 2.).

One can assume that the formation of erroneous disulfide at the active site of the reduced enzyme or modification of cysteine residues by quercetin should not influence diaphorase activity of glutathione reductase. We have expected that the enzyme will be able to keep full diaphorase activity in the presence of flavonoids. GR has been preincubated with NADPH for 1 min and quercetin or galangin were added. Both flavonoids had no significant effect on diaphorase activity (Fig. 3). DCIP was used as a substrate and because quercetin is able to reduce DCIP (B-ring of quercetin is oxidised to *o*-quinone, galangin does not react with DCIP) foremost the reduction of DCIP by quercetin was observed.

The GSSG-reductase activity was decreased by flavonoids while the diaphorase activity remained unaltered. We suppose that the inhibition by QU is a site-specific process and that exclude the enzyme FAD-domain, as indicated by unaltered GR diaphorase activity. Quercetin may react with sulfhydryl groups of vicinal cysteine residues (GRH2) and our results (decrease of inhibition by addition of NAC) may be in line with the formation of QU-thiol conjugates under conditions of catalysis (Fig. 2). The irreversible inhibition of GR by flavonoids has been demonstarted by Zhang et al.[10]

The cytotoxic action of quercetin was suspected to result from its pro-oxidant ability[21] and Metodieva et al.[8] suggested that DT-diaphorase is involved in prooxidant action of quercetin. Flavonoids did not alter diaphorase activity of GR, therefore the role of glutathione reductase in the depletion of NADPH during incubation of cells with some flavonoids should be considered.

ACKNOWLEDGEMENT

This work was supported by the grant of the Slovak Grant Agency number 1/1173/04.

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