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INHIBITORY EFFECT OF *N*-ETHYLMALEIMIDE IN TWO TYPES OF GLUTATHIONE REDUCTASES

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Glutathione reductase (GR) is a key enzyme of glutathione metabolism. This enzyme catalyzes the NADPH-dependent reduction of glutathione disulfide to a reduced form. The aim of the described study was to estimate an enzyme inhibition in two types of glutathione reductases (human and yeast) through N-ethylmaleimide (NEM). The glutathione reductase activity was determined by the spectrophotometric method based on the measurement of an absorbance decline (λ = 340 nm) due to oxidation of NADPH. Interestingly, it was found that the presence of 100 μM NEM had no effect in the two glutathione reductases. The inhibitory effect was proved in higher concentrations of N-ethylmaleimide; however, neither 2 mM NEM was able to diminish GR activity. The enzyme activity was reduced in both GRs; the human GR was inhibited by 15 % and 37 % in the presence of 1 mM and 2 mM NEM, respectively; the yeast GR was inhibited at the same concentrations of N-ethylmaleimide by 16 % and 35 %, respectively. We assessed NEM-induced inhibition of the enzyme activity in the presence of 1 mM GSSG (glutathione disulfide) where both GRs were inhibited to a larger extent than in 9 mM GSSG. On the other hand, if glutathione reductase was incubated with

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NADPH, followed by addition of NEM, the enzyme activity disappeared to a much higher extent. After 2 minutes of incubation with NADPH, the activity of yeast glutathione reductase was inhibited by 70 % and 100 % in the presence of 0.1 mM and 1 mM NEM, respectively.

Introduction

Glutathione is the most abundant low molecular weight thiol that is involved in intracellular antioxidant defense [1-3]. Glutathione occurs in two free forms — reduced as a thiol (GSH) and oxidized as glutathione disulfide (GSSG). Under the physiological conditions, over 99 % of glutathione is present in the reduced form in the cell [4-8]. Glutathione reductase (GR) is a crucial enzyme involved in glutathione metabolism. It occurs mainly in cytosol and mitochondria. Glutathione reductase exists as a homodimeric protein composed of two identical subunits containing 1 molecule of tightly-bound FAD (flavin adenine dinucleotide) per unit. It was proved that GR consists of six cysteine residues per subunit [9]. Two of the cysteine residues in each subunit form a redox active site, which is necessary for enzyme activity. Each subunit has the molecular weight about 55 kDa [3,9]. The mechanism of GR activity involves the transfer of electrons from NADPH (reduced nicotinamide adenine dinucleotide phosphate) through FAD to the disulfide bond in the active site. Glutathione reductase catalyzes the NADPH-dependent reduction of GSSG back to the reduced form. A decrease in the glutathione reductase activity can lead to the depletion of reduced glutathione, a rapid increase in the oxidized glutathione level and, therefore, to an increased oxidative stress in the cell [3,10,11].

The aim of this communication was to estimate the inhibitory effect of *N*-ethylmaleimide on the glutathione reductase activity. Generally, *N*-ethylmaleimide is an alkylating agent and is recognized as a potential inhibitor of GR activity [12-15]. However, our preliminary results showed that NEM was not able to inhibit glutathione reductase directly. Therefore, we aimed to assess the inhibitory effect in two types of glutathione reductases in detail.

Materials and Methods

Chemicals

Glutathione reductase (type III, from *Saccharomyces cerevisiae)*, glutathione reductase (human, recombinant), reduced glutathione, glutathione disulfide, NADPH, *N*-ethylmaleimide and potassium phosphate were purchased from Sigma-Aldrich (USA).

Determination of Glutathione Reductase Activity

The glutathione reductase activity was determined by the spectrophotometric method. This method is based on the measurement of an absorbance decline (λ = 340 nm) due to oxidation of NADPH [16]. The activity of both yeast and human glutathione reductases was determined at the temperature of 25 °C in 0.1 M potassium phosphate buffer (pH = 7.5) in 96-well plates [17]. We used two substrates, i.e., glutathione disulfide and NADPH. The decline of absorbance was monitored during 10 min. All the measurements were performed using a well-plate reader Tecan Infinite M200 (Austria). The results were presented as mU ml^{-1}; one Unit (U) was defined as the amount of enzyme that catalyzes the reduction of 1 μmol GSSG per min at pH = 7.5 (t = 25 °C). The molar extinction coefficient of NADPH (λ = 340 nm) was 6220 M^{-1} cm^{-1}.

GR Inhibition in Presence of *N*-ethylmaleimide

The inhibition of both yeast and human glutathione reductase through *N*-ethylmaleimide was assayed. NEM was diluted with distilled water to obtain the concentration of 220 mM. An inhibitory effect was investigated in the concentration limits of 0.1-2 mM NEM. 50 μl GR (yeast or human), 25 μl GSSG (1 mM or 9 mM) and 10 μl NEM were added. The measurement started with the addition of 25 μl NADPH (0.9 mM) and was monitored spectrophotometrically. The control samples were prepared using the same protocol but distilled water (10 μl) was added instead of NEM.

The inactivation of GR in the presence of NEM and NADPH was also tested. The mixture consisted of GR (50 μl; yeast GR 100 mU ml^{-1}), NEM (10 μl; 0.1 and 1 mM) and NADPH (25 μl; 0.8 mM). The mixture was incubated for 2 and 10 min on ice. The control samples were prepared by the identical protocol but distilled water (10 μl) was added instead of NEM. After the incubation, the measurement started with the addition of 25 μl GSSG (9 mM) and was monitored spectrophotometrically at the above-mentioned conditions. The values in the brackets mean the final concentrations of the compound in well.

Statistical Analysis

All the experiments were repeated at least two times. The results were processed by a one-way ANOVA test followed by the Tukey–Kramer test; $p > 0.05$ was considered as significant. The tested samples with NEM were compared with control ones. The results are presented as the mean value ± *SD*.

Results and Discussion

N-Ethylmaleimide was tested as a potential inhibitor of the glutathione reductase activity. *N*-Ethylmaleimide is an alkylating agent reacting with sulfhydryl moieties and forming stable thioether bonds [13,15]. This compound was used to diminish reduced glutathione levels in glutathione disulfide assays, because NEM reacts with GSH at pH < 7 [12,13]. On the other hand, *N*-ethylmaleimide was recognized as a typical inhibitor of glutathione reductase, so that its use in enzymatic assay of glutathione was unadvisable [14,18-20]. A possible inhibitory effect of NEM was estimated in two types of glutathione reductase, i.e., yeast and human. Interestingly, it was found that 100 μM NEM caused no inhibition of the yeast GR activity. In order to prove this finding, 100 μM NEM also in human GR was assessed and no significant decrease in the enzyme activity was found either (Fig. 1).

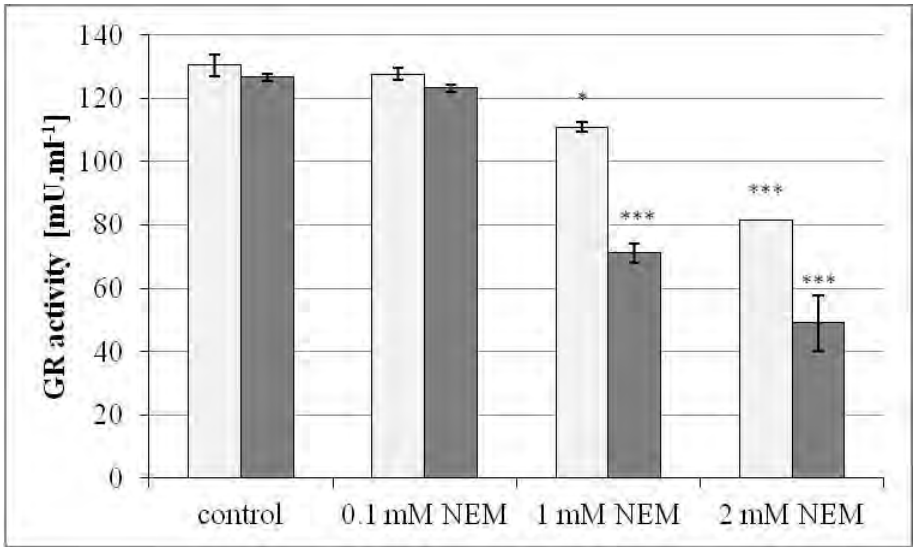

Fig. 1 Estimation of human glutathione reductase activity in the presence of *N*-ethylmaleimide. Glutathione reductase (150 mU ml^{-1}) was incubated with NEM (0.1 mM, 1 mM and 2 mM), glutathione disulfide (1 mM GSSG – gray columns; 9 mM GSSG – white columns) and NADPH (0.9 mM). Parameters of assay: λ = 340 nm; 10 min; 25 °C. Data are expressed as mean ± *SD*. (n = 2); *, $p < 0.05$; ***, $p < 0.001$ (compared with control at appropriate GSSG concentration)

The inhibition of the glutathione reductase activity was also found at higher concentrations of NEM in both GRs; however, even the presence of 2 mM NEM did not decrease the GR activity under 40 % of the test activity. In the presence of 1 mM and 2 mM NEM, human glutathione reductase was inhibited by 15 % and 37 %, respectively (with 9 mM GSSG). Yeast GR was inhibited at the same concentrations of *N*-ethylmaleimide by 16 % and 35 %, respectively (Fig. 2). We also assessed NEM-induced inhibition of the enzyme activity in the presence of 1

mM GSSG where both types of GR were inhibited to a larger extent than in 9 mM GSSG; in 2 mM NEM, the enzyme activity in yeast and human GR was decreased by 56 % and 61 % , respectively (Figs 1 and 2).

The obtained results concerning the effect of *N*-ethylmaleimide are in the contrast with those obtained by Griffith [14]. His results showed that 10 μM NEM caused nearly 35 % inhibition and 100 μM NEM was able to inhibit the glutathione reductase activity by 99 %.This discrepancy can be attributed to the different assay conditions, i.e., different GSSG levels and a type of glutathione reductase. Griffith used 100 μM NEM to assess 1.5 nM GSSG levels. In addition, GR was firstly incubated with NADPH according to the experimental protocol and the measurement started with the addition of GSSG. These conditions might lead to a reduction of cysteine in the active sites in enzymes and the following inhibition through NEM [14].

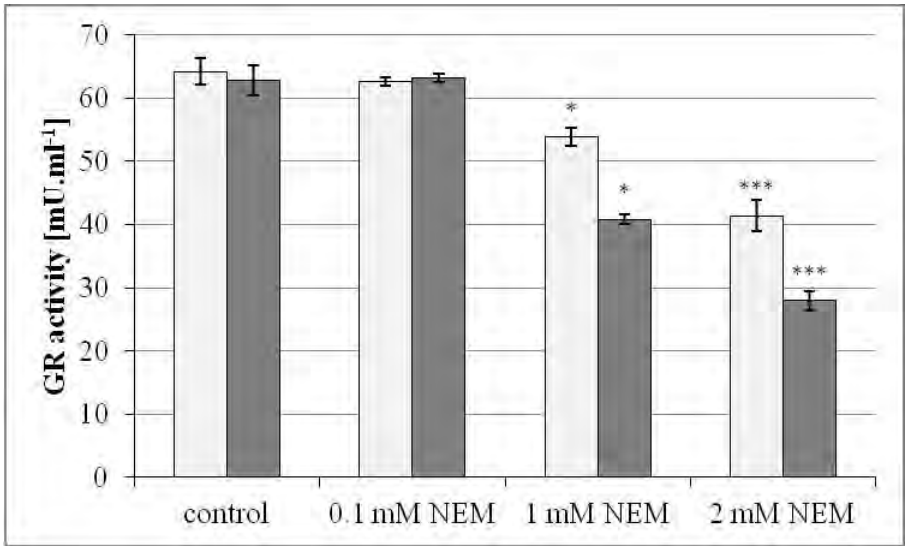

Fig. 2 Estimation of yeast glutathione reductase activity in the presence of *N*-ethylmaleimide. Glutathione reductase (70 mU ml^{-1}) was incubated with NEM (0.1 mM, 1 mM and 2 mM), glutathione disulfide (1 mM GSSG – gray columns; 9 mM GSSG – white columns) and NADPH (0.9 mM). Parameters of assay: λ = 340 nm; 10 min; 25 °C. Data are expressed as mean ± SD. ($n = 2$); *, $p < 0.05$; ***, $p < 0.001$ (compared to control at appropriate GSSG concentration)

Therefore, we finally tested an effect of incubation of GR with NADPH. After 2 min of incubation, the activity of glutathione reductase was inhibited by 70 % and 100 % in 0.1 mM and 1 mM NEM, respectively. The activity of yeast glutathione reductase was 98.0 ± 2.2 mU ml^{-1} (the control activity was 98.8 ± 1.5 mU ml^{-1}) in the presence of 0.1 mM NEM. After ten-minute incubation, the activity of GR was indetectable also in 0.1 mM NEM. The results showed that the presence of NADPH significantly influences the rate of enzyme inhibition due to the reaction of NEM with sulfhydryl groups.

Conclusion

In can be concluded that the rate of GR inhibition in the presence of NEM is dependent upon the GSSG level, and especially upon the presence of other substrate, NADPH. If the measurement started with the addition of NADPH; e.g., human glutathione reductase was inhibited by 15 % and 37 % in the presence of 1 mM and 2 mM NEM, respectively (with 9 mM GSSG). The enzyme activity of both types of GR in the presence of 1 mM GSSG was inhibited to a larger extent than in 9 mM GSSG. On the other hand, if glutathione reductase was first incubated with NADPH and the reaction started with the addition of GSSG, the GR activity was inhibited through NEM in a few minutes.

Abbreviations

NEM, *N*-ethylmaleimide; GR, glutathione reductase; GSH, glutathione (reduced form); GSSG, glutathione disulfide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide.

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