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Homocysteine effects on cellular glutathione peroxidase (GPx-1) activity under *in vitro* conditions

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Abstract
Hyperhomocysteinemia is associated with a lot of diseases including cardiovascular diseases and neural tube defect, but it has not been clarified exactly which mechanism is responsible for occurence disease. Here, homocysteine (Hcy) and cysteine (Cys), which are thiol containing amino acids, were examined for their effect on glutathione peroxidase (GPx) activity. İt was observed that the GPx-1 activity was inhibited under severe hyperhomocysteinemia (50–500 μM Hcy) conditions, especially at low glutathione concentrations but that cysteine increased GPx-1 activity at low glutathione concentrations and inhibition clearly appeared at 500 μM Cys concentration.

Keywords: *Glutathione peroxidase, homocysteine, cysteine, glutathione reductase*

Abbreviations: *FAD^+, oxidized flavine adenine dinucleotide phosphate; GPx, glutathione peroxidase; GPx-1, cellular glutathione peroxidase; GPx-2, gastrointestinal glutathione peroxidase; GPx-3, plasma glutathione peroxidase; GPx-4, phospholipid hydroperoxide glutathione peroxidase; GPx-5, epididymal glutathione peroxidase; GPx-6, Glutathione peroxidase 6; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; Hcy, homocysteine; NaP, sodium phosphate; ROS, reactive oxygen species; TBH,* tert-*butylhydroperoxide*

Introduction

Homocysteine (Hcy) is a sulphur containing, non-essential amino acid biosynthesized from dietary methionine [1]. It is present in plasma in several different forms. Approximately 70% is bound to plasma proteins, mainly albumin, by a disulphide link. The remaining homocysteine combines with other thiols, including cysteine, resulting in homocysteine-cysteine mixed disulphide (the most abundant disulphide species), and homocysteine itself, to form the dimer homocystine. Only a small proportion (approximately 1%) normally circulates as the free thiol compound. The different forms of homocysteine appear to be in a state of flux exchanging readily between the different forms [2].

Homocysteine may either be catabolized to cysteine or remethylated to methionine [3]. Under conditions of low protein intake, homocysteine is metabolised primarily to methionine by a remethylation pathway. When the remethylation pathway is saturated, or when cysteine is required, homocysteine is converted to cystathionine (and then cysteine) by cystathionine β-synthase. Vitamine B_6 (pyridoxine) is an essential cofactor. Cysteine may be metabolised further to sulphate and excreted in the urine [4].

Available results suggest that almost all human tissues have some capacity to convert methionine to homocysteine but that the apportionment of homocysteine to the transsulfuration or remethylation pathways may vary markedly from tissue to tissue. Because fetal tissues and placenta lack γ-cystathionase activity, it has been suggested that cyst(e)ine may be an essential amino acid at this stage of life. By 30 weeks gestation, hepatic γ-cystathionase

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attains 23 percent of the adult specific activity; by full term (39 weeks), 46 percent. The specific activity continues to rise postnatally for about 1 year [5].

In the adult population, the normal plasma total homocysteine is 5–15 μmol/L, with a mean concentration of about 10μmol/L. Hyperhomocysteinemia is usually defined as a plasma tHcy > 15μmol/L, and is denoted moderate (15–30 μmol/L), intermediate (30–100 μmol/L) or severe hyperhomocysteinemia (> 100 μmol/L) [6].

An elevated plasma level of homocysteine is a common, independent risk factor for cardiovascular disease [7]. Hyperhomocysteinemia is also associated with thrombosis, stroke [8,9], cerebrovascular disease [10], Alzheimer's disease [11,12], neural tube defect [13], placental abruption/infarction and pre-eclampsia [14,15,16]. But the mechanisms responsible for the occurence of the disease are still incompletely understood.

One commonly held view is that oxidative stress may be an important contributing factor [17]. Autooxidation of homocysteine in vitro generates reactive oxygen species (ROS), including hydrogen peroxide and superoxide, and promotes oxidation of low density lipoprotein. Whether or not homocysteine auto-oxidation is a major mechanism for the generation of ROS *in vivo* is uncertain. Conversion of homocysteine to it's disulfide forms in plasma is mediated mainly by thiol-disulfide exchange reactions rather than by copper-dependent oxidation, which suggests that homocysteine is unlikely to be a major source of hydrogen peroxide *in vivo*. Therefore, it is perhaps more likely that indirect mechanisms are responsible for the oxidative stress of hyperhomocysteinemia. Indirect oxidative effects of hyperhomocysteinemia may include generation of superoxide from xanthine oxidase or uncoupled endothelial nitric oxide synthase, downregulation of antioxidant enzymes (e.g.GPx-1) and depletion of intracellular glutathione [18].

In some studies it has been indicated that homocysteine inhibits the expression of cellular glutathione peroxidase (GPx-1) [19,20,21], which can lead to an increase in ROS and which would alone induce oxidative stress. Dayal et al. demonstrated that deficiency of GPx-1 exacerbates endothelial dysfunction in mice with moderate hyperhomocysteinemia [18]. These *in vivo* findings provide support for the hypothesis that indirect oxidative mechanisms are responsible for the disease formation in hyperhomocysteinemia.

However, the questions of if and how homocysteine induces oxidative stress are not yet clear. In this study we examine the of effects homocysteine on GPx-1 activity, which is an antioxidant enzyme. And also we examine the effects of cysteine on this enzyme to compare with homocysteine effects.

Materials and methods

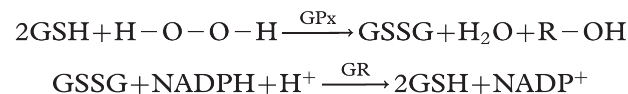
Materials

Glutathione peroxidase (G-6137), glutathione reductase (G-3664), L-cysteine (C-7352), DL-homocysteine (H-4628), β-NADPH (N-7505), FAD (F-6625), reduced glutathione (G-4251) and oxidized glutathione (G-4626) were purchased from Sigma and *tert*-butyl hydroperoxide (tBH, 820244) was purchased from Merck.

Biochemical measurements

GPx activity measurement. GPx activity measurements were carried out according to the Beutler method [22].

This method is a coupled, kinetic, spectrophotometric assay in which the incubation mixture containing Tris- HCl (1M, pH 8.0), GSH (0.1–0.25–0.5–0.75–1.0 mM), GR(1 U/mL), NADPH (0.2 mM), GPx-1 (5 mU/mL) and H_2O_2 were preincubated at 37°C for 10 min. Then the t-BH (0.07 mM) was added and the decrease in absorbance at 340 nm was determined.



$$2GSH + H-O-O-H \xrightarrow{GPx} GSSG + H_2O + R-OH$$
$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

Determination of enzyme concentration. To determine the appropriate enzyme concentration for this study the absorbance changes at 340 nm during 10 min were recorded in the presence of different GPx-1 activity (50–0.01 mU/mL). At the end of this study, considering the case of application, it was concluded that 5 mU/mL GPx-1 was the most appropriate concentration.

GR activity measurement. GR activity was determined by a modification of the procedure of Beutler [22]. GR activity catalyzes the reduction of oxidized glutathione (GSSG) by NADPH or NADH to reduced glutathione (GSH). The activity of the enzyme is measured by following the oxidation of NADPH spectrophotometrically at 340 nm.

$$NAD(P)H + H^+ + GSSG \xrightarrow{GR} NAD(P)^+ + 2GSH$$

GR is a flavin enzyme and the complete activation of apoenzyme requires the preincubation of enzyme with FAD, necessarily done before GSSG and NADPH are added to the reaction system since these seem to interfere with activation of the enzyme by FAD. Reaction system containing Tris- HCl (1M, pH 8.0), GR (1U/mL), FAD (1 μM) and H_2O were incubated at 37°C for 10 min. Then GSSG (0.5 mM) was added

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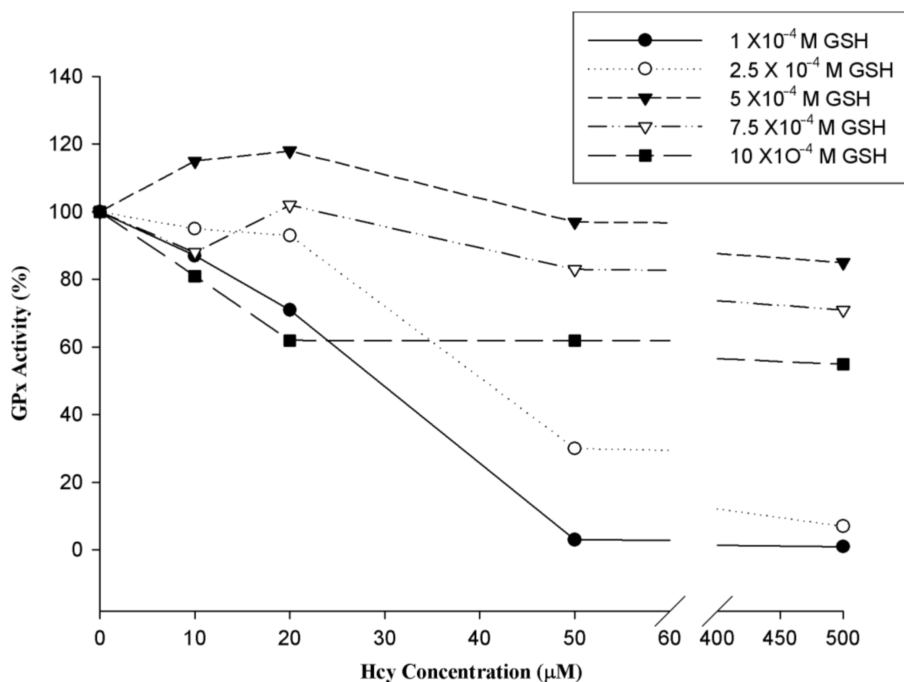

Figure 1. Hcy effect on bovine GPx-1 activity.

and the mixture incubated at 37°C for 10 min. Finally NADPH (0.2 mM) was added and the decrease in absorbance at 340 nm was determined.

Inhibition experiments. Hcy and Cys, which are thiol-containing amino acids, were used as inhibitors in our experiments. Inhibition experiments were carried out at different GSH concentrations ($1x10^{-4}$ M, $10x10^{-4}$ M GSH) in the presence of 10 μM, 20 μM, 50 μM, 500 μM inhibitor (Hcs or Cys) concentration. 5 mU/mL GPx-1% activity changes are shown in Figures 1 and 2.

To investigate the effects of Hcy and Cys on GR (10 U/mL) activity we examined the enzyme activity at 0μM, 50 μM, 500μM inhibitor concentrations in the presence of $5x10^{-4}$M GSSG. Hcy effect on GR activity is shown in Table I and Cys effect is shown in Table II.

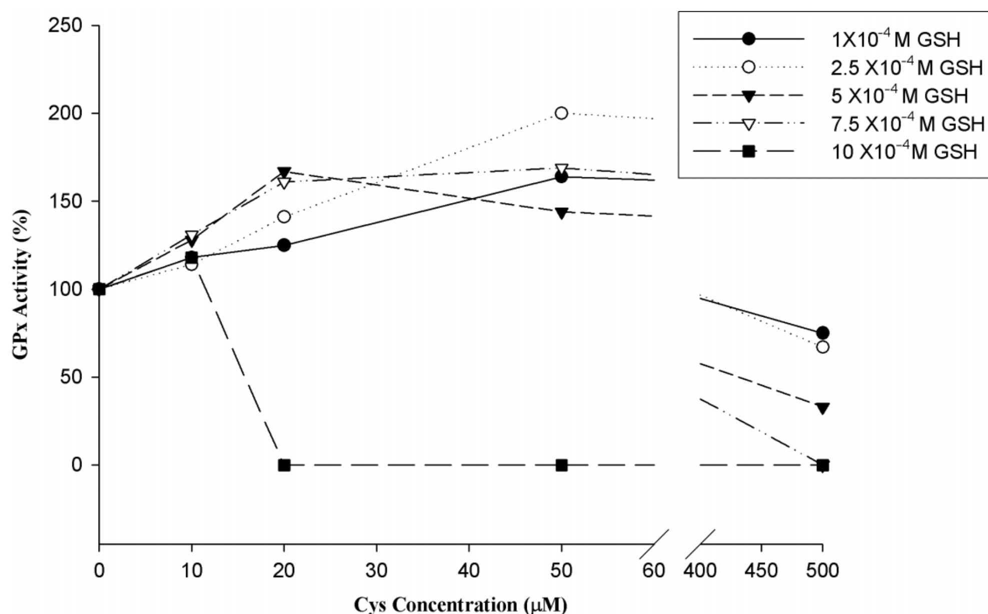

Figure 2. Cys effect on bovine GPx-1 activity.

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Table I. Hcy effect on GR Activity.

Hcy concentration	GR Activity (U/mL)	
	With FAD (1 μM)	Without FAD
0 μM Hcy	1.28	0.68
50 μM Hcy	1.74	1.49
500 μM Hcy	1.58	1.30

Table II. Cysteine effect on GR activity.

Cys concentration	GR Activity (U/mL)	
	With FAD (1μM)	Without FAD
0 μM Cys	1.28	0.68
50 μM Cys	1.07	0.70
500 μM Cys	1.66	0.84

Experiment of NADPH consumption. Another important aspect of the work was to investigate whether or not NADPH was consumed by homocysteine's itself. For this purpose the absorbance change was recorded of NADPH (2 mM, 100 μL) in sodium phosphate (NaP) buffer (pH 7, 5 mM, 900 μL) at 340 nm during 10 min and compared with the absorbance changes of mixtures which contained NaP buffer, NADPH (2 mM, 100 μL) and Hcy at different concentrations (50 μM, 500 μM, 1 mM, 1.5 mM, 2 mM) at 340 nm during 10 min. The results are summarized in Table III.

In all measurements pure water was used as blank and measurements were repeated three times.

Results

According to Figure 1 Hcy inhibits GPx-1 activity in hyperhomocysteinemia conditions (50–500 μM Hcy).

The results in Figure 2 showed that Cys increased GPx-1 activity in low glutathione concentrations, and inhibition clearly appeared at 500 μM Cys concentration.

The results in Table I showed that Hcy had no inhibitory effect on GR activity and actually increases enzyme activity.

The results in Table II showed that Cys had no inhibitory effect on GR activity.

Table III showed that the end of this experiment, NADPH was not consumed by homocysteine's itself.

Discussion

Glutathione peroxidases (Gpxs) are selenoproteins [23,24], containing Se in the form of the amino acid selenocysteine at their active site [25,26]. GPxs catalyze the reduction of hydrogen peroxide and various organic hydroperoxides into water and alcohols, respectively [27]. There are at least six GPx isoenzymes found in mammals; 1) cellular glutathione peroxidase (GPx-1) [28], 2) gastrointestinal glutathione peroxidase (GPx-2) [29], 3) plasma glutathione peroxidase (GPx-3) [30], 4) phospholipid hydroperoxide glutathione peroxidase (GPx-4) [31], 5) epididymal glutathione peroxidase (GPx-5) [32], and, 6) Glutathione peroxidase 6 (GPx-6) [33].

GPx-1 is a cytosolic enzyme expressed in every cell type and is thought to be one of the major antioxidant proteins in mammals [23]. The enzyme was first described in 1957 and is found mainly in cytoplasm [28].

The mechanism for the GPx reactions is shown in Figure 3. The first step is an oxidation of the selenol group of the enzyme by a hydroperoxide to form a selenic acid derivative (ESeOH). The second step leads to the formation of covalent bonding between the sulfur of the GSH and the selenium of the enzyme to form a selenenyl sulfide adduct (ESeSG). The last step is the regeneration of the reduced enzyme via a second GSH that breaks the selenadisulfide bridge in ESeSG [34] (see Figure 3).

In previous studies, it has been shown that homocysteine inhibits the expression of GPx-1 [19,20,21] but no study exists concerning the direct effects of Hcy on GPx-1 activity. The results of the present study indicate that high Hcy concentrations (50–500 μM), especially at low glutathione concentrations, inhibit GPx-1 activity. These observation can be a step at explaning of the responsible mechanism of Hcy on antioxidant system such as atherosclerosis formation at the endothelial bed.. The red cells of normal adults contain 68.5 ± 10.8 mg/dL GSH [22]. In our inhibition experiments we used low GSH concentrations to examine the effects of the thiol containing amino acids, Hcy and Cys, at low antioxidant concentrations.

Besides Hcy we also examined the effects of cysteine to compare with Hcy. Hcy inhibited GPx-1 activity in hyperhomocysteinemia conditions (50–500 μM Hcy). On the other hand cysteine increased GPx-1 activity in low glutathione concentrations, and it's inhibition clearly appeared at 500 μM Cys concentration.

Table III. Investigation of the whether or not NADPH is consumed by homocysteine itself at 340 nm

Hcy Conc.	0 μM	50 μM	500 μM	1 mM	1.5 mM	2 mM
Δ OD/min.	0.0026	0.0030	0.0026	0.0023	0.0030	0.0024

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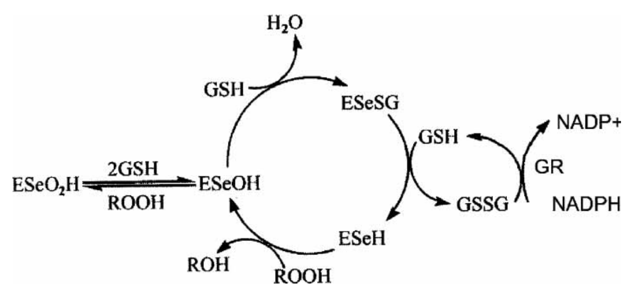

Figure 3. The catalytic mechanism of natural GPx [34,35].

These results can be explained by two possible mechanism; 1) In high concentrations, thiol metabolites (Hcy, Cys) are bound to Se, which is the active site of the enzyme, directly and block ESeSG formation in the reaction cascade so that the activity of the enzyme is decreased 2) Thiol metabolites can react with GSH in the last step of the GPx reaction so that the enzyme cannot be regenerated and activity is decreased.

GPx activity measurement were carried out in two stages. In the first stage; GPx detoxifies the hydroperoxide with the aid of GSH; in this step GSH is converted to GSSG. In the second stage GR reduces the GSSG by using NADPH as a cofactor. Hydroperoxide reduction was followed by a decrease in NADPH absorbance at 340 nm.

To investigate the effect of Hcy on GR activity we examine the enzyme activity at 0μM, 50 μM, 500μM Hcy concentrations. In the absence of Hcy (0μM Hcy) GR activity was 1.28 U/mL − 0.68 U/mL (with FAD-without FAD), under the conditions of 50μM and 500μM Hcy concentrations, GR activity was found 1.74 U/mL − 1.49 U/mL (with FAD-without FAD), 1.58 U/mL − 1.30 U/mL (with FAD-without FAD) respectively. These results showed that Hcy has no inhibitor effect on GR activity and actually increases enzyme activity. In consequence of this study it is demonstrated that Hcy inhibits GPx activity directly.

Another important aspect of the work was to investigate whether or not NADPH was consumed by homocysteine itself. For this purpose we recorded the absorbance change of NADPH in NaP buffer at 340 nm during 10 minutes in comparison with the absorbance changes of mixtures which contained NaP buffer, NADPH and Hcy at different concentrations over the same period. At the end of the study it was observed that Hcy alone did not consume NADPH.

In conclusion, all these observations indicated that Hcy inhibits GPx-1 activity directly in severe hyperhomocysteinemia (50–500 μM Hcy) conditions especially at low antioxidant status.

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