Free Rad. Res., Vol. 32, pp. 171–179
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Elevated Plasma Homocysteine Elicits an Increase in Antioxidant Enzyme Activity

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Accepted by Prof. B. Halliwell

(Received 24 May 1999; In revised form 22 July 1999)

Elevated plasma homocysteine is considered to be a risk factor for cardiovascular disease. The mechanisms for this effect are not fully understood but there is some evidence for a role for reactive oxygen species (ROS). This study was conducted to explore the effects of elevated plasma total homocysteine (tHcy) concentration on activity of antioxidant enzymes in the circulation. The study group consisted of 10 patients with inherited defects of homocysteine metabolism, from whom 41 blood samples were collected over a period of six months. Blood samples were also collected from 13 of their obligate heterozygous parents. For data analysis samples were classified as those with plasma tHcy $< 20\,\mu M$ or $\geq 20\,\mu M$. The activity of erythrocyte superoxide dismutase (SOD) and plasma glutathione peroxidase (GSHPx) was elevated in samples with plasma tHcy $> 20\,\mu M$. Moreover, a significant correlation was demonstrated between plasma GSHPx activity, plasma glutathione peroxidase protein and plasma tHcy. *In vitro* studies confirmed that this observation was not due to a simple chemical enhancement of enzyme activity. Homocysteine protected GSHPx from loss of activity following incubation at 37°C. A similar effect was seen with another thiol-containing amino acid, cysteine. Results suggest that elevated plasma tHcy represents an oxidative stress, resulting in an adaptive increase in activity of antioxidant enzymes in the circulation.

Keywords: Cardiovascular disease, homocysteine, endothelial dysfunction, superoxide dismutase, glutathione peroxidase

INTRODUCTION

Premature vascular disease and thrombotic complications in adolescence and in infancy have long been recognised as features of the rare inherited metabolic disorder homocystinuria, characterised by grossly elevated plasma homocysteine (typically $> 100\,\mu M$ at diagnosis; normal range 5–15 μM).[1] Recently a moderate elevation of plasma homocysteine concentration (15–50 μM), (hyperhomocysteinaemia) has been associated with an increased risk of premature cardiovascular disease in the general population.[2,3] Ten percent of the total population risk for cardiovascular disease has been attributed to hyperhomocysteinaemia.[4] Hyperhomocysteinaemia can be caused by a metabolic defect in either the transulphuration or remethylation

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pathways of homocysteine, or by deficiencies of the cofactors required for these pathways.

The precise mechanisms by which homocysteine promotes the development of vascular disease are unknown, but the endothelium is the focus of current research interest. Endothelial injury is a critical initiating event in the development of vascular disease,[5] and an elevation in the concentration of circulating homocysteine can cause perturbation of normal endothelial cell function. Impaired endothelial cell function has been demonstrated in children with homocystinuria,[6] and adults with moderately elevated plasma homocysteine.[7] These studies support the concept that endothelial dysfunction is an important component of the vascular effects of homocysteine.

Several factors may cause endothelial damage *in vivo*, including reactive oxygen species (ROS). In *in vitro* systems, thiol containing compounds such as homocysteine readily autoxidise to their disulphide form in the presence of cupric or ferric ions with the associated production of hydrogen peroxide and other ROS including the superoxide anion and the hydroxyl radical.[8–10] Several investigators have argued that homocysteine exerts its damaging effects on the vasculature through mechanisms involving ROS.[10–12] However, the majority of studies in this context have focused on the nature and extent of damage to endothelial cells in culture in response to non-pathophysiological concentrations of homocysteine. Certain antioxidants are reported to protect endothelial cells in culture from the toxic effects of homocysteine, and the effectiveness of different antioxidants has indicated the nature and source of ROS that may be involved in homocysteine-mediated endothelial damage.[11,13,14] Although informative, results of these *in vitro* studies need to be interpreted with caution and, where possible, supported by *in vivo* studies. If elevated homocysteine poses an oxidant threat *in vivo* there may be a compensatory mechanism to increase the level of antioxidant protection. One possible response would be an increased activity of antioxidant enzymes. If such an effect were demonstrated *in vivo*, it would support the argument for ROS being involved in homocysteine-linked vascular damage.

The present study was conducted to explore the effects of elevated plasma homocysteine concentration on the activity and concentration of the erythrocyte antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GSHPx), catalase and plasma GSHPx.

MATERIALS AND METHODS

Subjects

Our study group consisted of 10 patients (6 male and 4 female), mean age 26 years, range 6–66 years, with inherited defects of homocysteine metabolism. Forty-one blood samples were collected from these patients, following an overnight fast, as part of their routine monitoring. Single fasting blood samples were collected from 13 of their obligate heterozygous parents. The protocol for this study was approved by the South Sheffield Research Ethics Committee.

Sample Collection

Fasting venous blood samples were collected from the median cubital vein into lithium heparin tubes, placed on ice and centrifuged at 3000*g* for 10 min, at 4°C within 30 min of venepuncture. Plasma was aliquoted and stored at −70°C. Erythrocytes were washed three times with an equal volume of isotonic saline (0.9%), resuspended in an equal volume of distilled water and stored at −70°C.

Reagents

Homocysteine, cysteine, catalase, GSHPx, pyrogallol, DTPA and other reagents were obtained from Sigma Chemical Company (Poole, Dorset, UK). Standard solutions of cyanmethaemoglobin were obtained from BDH (Poole, Dorset, UK).

Biochemical Analyses

Plasma Total Homocysteine (tHcy)

Plasma tHcy (protein bound and free forms) was measured as previously described by Spaapen *et al.*[15] Plasma samples were treated with tri-*n*-butylphosphine to reduce disulphide bonds between homocysteine and plasma protein and between mixed disulphides. Liberated thiols were then derivatised using 7-fluorobenzo-2-oxa 1,3-diazole-4-sulphonic acid (SBD-F). Compounds were then separated by high performance liquid chromatography (HPLC) with fluorescence detection. This was performed using a Waters 600 pumping and controller system, Waters 717 autosampler and a Waters 474 fluorescence detector (Waters Company Millipore, Milford, MA, USA).

Erythrocyte Superoxide Dismutase (SOD)

Determination of erythrocyte SOD activity was performed using a method based on Marklund and Marklund,[16] adapted for use on the Cobas Bio-Autoanalyser (Roche Diagnostica, Welwyn Gardens, UK). The spectrophotometric method is based on the ability to inhibit the autoxidation of pyrogallol, which occurs spontaneously above pH 7.5, producing superoxide radicals and products which absorb at 420 nm. In order to extract SOD, 700 µl of cold distilled water, 125 µl of cold chloroform and 200 µl of ethanol were added to 100 µl of erythrocyte haemolysate. Samples were shaken for 15 min at 4°C and centrifuged at 3000*g* for 10 min at 4°C to remove denatured haemoglobin. Five different volumes of extract were incubated at 25°C with 245 µl of TRIS cacodylic acid buffer (50 mM TRIS/1 mM diethylenetriaminepentacetic acid (DTPA), pH 8.2 with cacodylic acid) and 3 µl of pyrogallol (20 mM in 11.5 mM HCl). The extent of autoxidation was calculated after 30 s of incubation, from the change in absorbance at 420 nm. One unit of SOD activity was defined as the amount of the enzyme inhibiting the autoxidation by 50%.

Erythrocyte Catalase

Catalase activity was determined in erythrocyte lysates by a coupled enzyme assay, which measures the increase in absorbance at 340 nm due to the production of NADH from the peroxidic reaction between ethanol, hydrogen peroxide and catalase. Hydrogen peroxide is produced as a substrate from the oxidation of glucose catalysed by glucose oxidase. Catalase then oxidises ethanol to acetaldehyde in the presence of hydrogen peroxide. Acetaldehyde is then oxidised by aldehyde dehydrogenase to produce acetate with concomitant reduction of NAD^+ to NADH. Standards were prepared from commercially available bovine liver catalase. The assay used was adapted for use on the Cobas Autoanalyser.[17]

Erythrocyte GSHPx

The activity of GSHPx in erythrocyte lysates was determined using a commercial kit (RS 506; Randox Laboratories, Crumlin, Northern Ireland). This assay is based on the method of Paglia and Valentine,[18] in which GSHPx catalyses the oxidation of glutathione using cumene hydroperoxide as the substrate. Oxidised glutathione is then used as a substrate for glutathione reductase, with the subsequent oxidation of added NADPH monitored as a decrease in absorbance at 340 nm. Samples were diluted with diluting buffer supplied by the manufacturer, to convert the GSHPx to the reduced form and Drabkins reagent (Sigma) added to inhibit other peroxidases present in the sample.[19]

Plasma GSHPx

Plasma GSHPx activity was measured as described above. Plasma samples were also diluted with the diluting reagent supplied by the manufacturer, to convert the GSHPx to the reduced form, with Drabkins reagent (Sigma) added to inhibit other interfering peroxidases present in the sample.[19] In addition, plasma GSHPx protein was measured using a commercial ELISA kit

(R&D systems, Abingdon, UK). The presence of plasma GSHPx protein was detected with a biotinylated-polyclonal antibody specific to human plasma GSHPx, using amplification by biotin–streptavadin, coupled to alkaline phosphatase. Alkaline phosphatase activity was determined measuring para-nitrophenyl-phosphate (pNPP) at 405 nm. The antibodies used in this assay system were obtained by using a synthetic antigen and have been purified by affinity chromatography.[20] Plasma GSHPx activity and enzyme protein were expressed as U/l and ng/ml respectively.

Haemoglobin (Hb) Concentrations

Erythrocyte enzyme activity was expressed as a function of haemoglobin (U/g Hb). Hb was determined using the cyanomethaemoglobin method described by Crosby and Houchin,[21] which has been adapted for use on the Cobas autoanalyser using cyanomethaemoglobin standards (57.2 mg/100 ml).

Statistical Analysis

Data were analysed using SPSS for Windows. The activities of erythrocyte SOD, catalase, and GSHPx, and plasma GSHPx, are presented as mean (±SEM). Comparison of enzyme activities was made using the two-sample *t*-test assuming unequal variances. Regression analysis was carried out to explore associations between each variable and tHcy.

Analysis of variance (ANOVA) was used to quantify the effects of time and concentration of homocysteine on GSHPx activity, followed by a Scheffé test where significant differences were detected. Statistical significance was accepted at the 95% confidence level ($P \leq 0.05$).

RESULTS

In-house quality control material was used with every batch of analyses. The inter-batch coefficient of variation was: erythrocyte SOD, 3.6%; erythrocyte catalase, 3.1%; erythrocyte GSHPx, 4.4%; plasma GSHPx (Randox method), 2.8%; plasma GSHPx (ELISA method), 5.6%.

Effects of Plasma tHcy on Enzyme Activity

For data analysis samples were classified into those in which the plasma tHcy concentration was $< 20\,\mu M$ or $\geq 20\,\mu M$. The cutoff was derived from the mean ± 2SD of data obtained from 13 obligate heterozygous controls.

Table I shows the mean (±SEM) of erythrocyte SOD, catalase and GSHPx, and plasma GSHPx activity in samples with plasma tHcy $< 20\,\mu M$ compared with plasma tHcy $\geq 20\,\mu M$. The mean plasma tHcy (±SEM) for each group was 12.7 μM (±0.91) and 112.8 μM (±11.02) respectively.

Neither erythrocyte catalase nor GSHPx activity were different between the two groups. The erythrocyte SOD activity was elevated in samples with plasma tHcy $\geq 20\,\mu M$ when compared with samples with a plasma tHcy $< 20\,\mu M$ ($P = 0.0002$). Similarly, plasma GSHPx activity was significantly greater ($P < 0.02$) in samples with elevated plasma tHcy when compared with samples with plasma tHcy $< 20\,\mu M$.

Linear regression analysis of erythrocyte SOD activity against plasma tHcy showed a significant positive correlation ($P < 0.005$) (Figure 1). Erythrocyte CuZn SOD activity has been reported to decrease as a function of age[22,23] and this

TABLE I The effect of tHcy on activities of erythrocyte SOD, catalase, GSHPx and plasma GSHPx

Plasma tHcy (μM)	Mean (SEM) erythrocyte antioxidant enzyme activity (U/g Hb)			Plasma antioxidant activity (U/l)
	SOD	Catalase	GSHPx	GSHPx
< 20 ($n = 17$) 12.7 (0.91)	5098 (122.7)	194 (5.7)	34 (2.7)	781 (45.1)
≥ 20 ($n = 37$) 112.8 (11.02)	5699** (90.0)	204 (4.2)	35 (1.2)	906* (31.4)

Significantly different from group with tHcy $< 20\,\mu M$ (Student's *t*-test). $^{**}P < 0.0002$; $^{*}P < 0.02$.

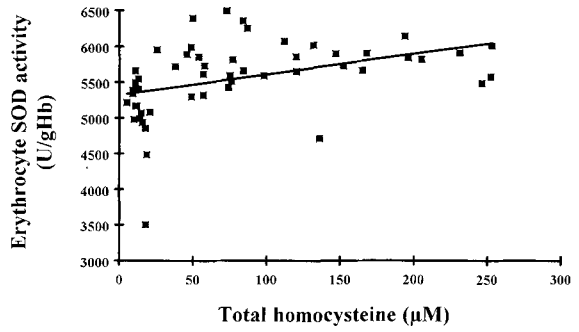

FIGURE 1 Association between erythrocyte SOD activity and plasma total homocysteine ($r = 0.404$, $n = 54$, $P < 0.005$).

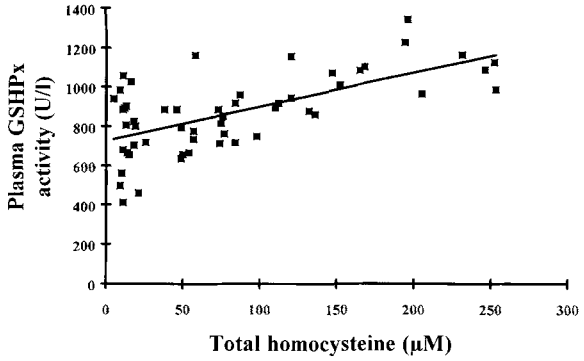

FIGURE 2 Association between plasma GSHPx activity and plasma total homocysteine ($r = 0.639$, $n = 54$, $P < 0.001$).

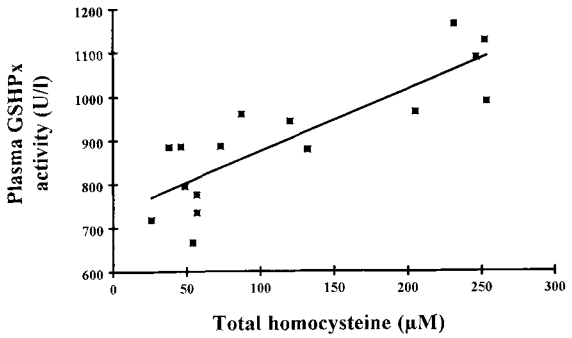

FIGURE 3 Association between plasma GSHPx activity and plasma total homocysteine in a patient with homocystinuria ($r = 0.844$, $n = 16$, $P < 0.001$). Samples were collected over a six month period.

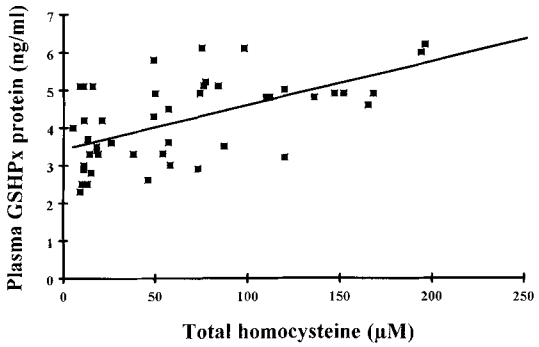

FIGURE 4 Association between plasma GSHPx protein and plasma total homocysteine ($r = 0.576$, $n = 48$, $P < 0.001$).

relationship was observed in this dataset, taking the baseline measurement per subject ($r = 0.544$; $p = 0.0073$). Having corrected for an effect of age on erythrocyte SOD activity the independent effect of tHcy just failed to reach statistical significance ($P = 0.0721$).

Plasma GSHPx activity was strongly correlated with plasma tHcy ($P < 0.001$) (Figure 2) but no effect of age was seen ($P = 0.1907$). Serial samples ($n = 16$) taken from a single patient with homocystinuria from a period of poor compliance and control to a period of better control showed a particularly strong correlation ($P < 0.001$) (Figure 3).

There are three distinct isozymes of GSHPx, endothelial, erythrocyte and plasma.[12] Plasma GSHPx is found in low concentrations compared with the erythrocyte and endothelial form, and little is known regarding its origin. The levels of activity for plasma GSHPx are very similar to those reported by Guemori *et al.*[22] using the same assay. The Randox method used for GSHPx activity is not specific for any form of GSHPx. In order to determine whether the increased activity of plasma GSHPx was due to an increase in the amount of enzyme, an ELISA specific for the plasma form of human GSHPx was performed. Samples with plasma tHcy $\geq 20\,\mu M$ had a mean (±SEM) GSHPx protein of 4.55 ng/ml (±0.18) compared with 3.48 ng/ml (±0.23) ($P = 0.0004$) in samples with a plasma tHcy $< 20\,\mu M$. A strong positive correlation was observed between GSHPx protein and plasma tHcy ($P < 0.001$) (Figure 4).

Effect of Homocysteine on the Activity of Plasma GSHPx and Purified Bovine Erythrocyte GSPHx *In Vitro*

The observed relationship between plasma GSHPx enzyme activity, protein and plasma total homocysteine support a role for ROS in the mechanism of the effect of homocysteine. However previous workers have suggested that homocysteine inhibits GSHPx.[24] In order to clarify the role of homocysteine in eliciting an increase in activity of plasma GSHPx observed in this human study we explored whether this was an effect that could be reproduced *in vitro*, which would imply a direct chemical enhancement of enzyme activity. Plasma was incubated at 37°C over a period of 3 h with a final concentration of added homocysteine between 50 and 1000 μM. Samples were incubated in triplicate, and aliquots removed at times 0, 0.5, 1.0, 1.5, 2.0 and 3 h. There was no significant effect of increasing homocysteine concentration on GSHPx activity ($P = 0.59$), but with increasing time of incubation, the mean (±SEM) activity at all concentrations of homocysteine fell from 441 (±8.2) U/l at time 0 to 400 (±5.9) U/l at 3 h ($P = 0.025$). In a separate experiment the incubation time was extended to 24 h, and measurements made at 0, 4.0 and 24 h. Samples were incubated in duplicate and the mean calculated. Added homocysteine had a protective effect on GSHPx activity (2-way ANOVA, $P < 0.0001$) (Figure 5).

The acute effects of homocysteine were studied by adding homocysteine immediately prior to measuring GSHPx activity, after a 24 h incubation of plasma without homocysteine. Activity in the control sample decreased by 85% compared with an average of 42.5% in samples to which homocysteine (50–1000 μM) had been added ($P < 0.001$), confirming the protective effect of homocysteine.

A similar investigation was carried out using purified bovine erythrocyte GSHPx to allow comparison with data from Nishio *et al.*[24] Purified bovine erythrocyte GSHPx, at a concentration of 2 U/ml (Sigma units), which corresponded to approximately 750 Randox units/l, was pre-incubated with homocysteine or cysteine at a concentration of 0–1000 μM for 0–6 h at 37°C. The activity was measured using the usual protocol at hourly intervals. GSHPx activity in the control sample decreased from 774(±19.7) U/l to 178.5(±16.3) U/l ($P < 0.0001$) over a 6 h incubation period (Figure 6). In contrast, the mean (±SEM) fall in GSHPx activity in samples incubated with homocysteine was 25 (±3.6%). The difference was highly statistically significant ($P < 0.0001$).

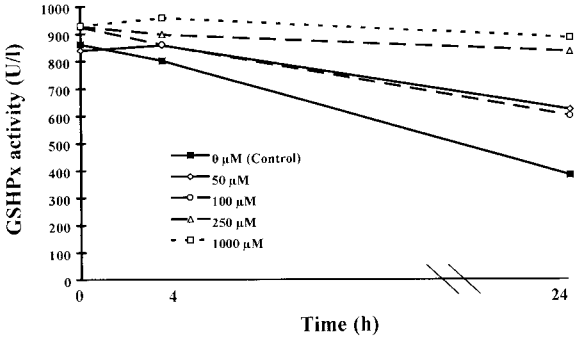

FIGURE 5 Effect on GSHPx activity of incubating plasma with different concentrations of homocysteine. (Each value represents the mean of duplicate samples.) Fall in activity was greatest in the absence of homocysteine ($P < 0.0001$).

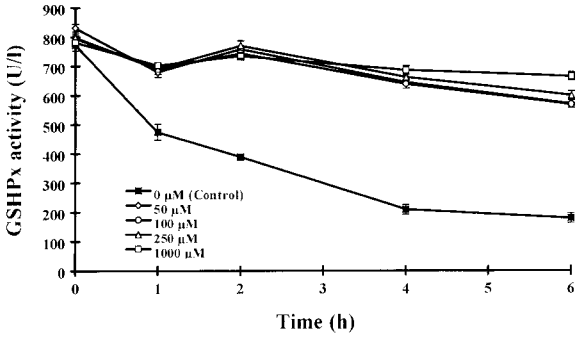

FIGURE 6 Effects on enzyme activity of incubating purified GSHPx with a range of different homocysteine concentrations. (Each value represents the mean of four samples ± SEM.) The mean fall in activity was greater in samples not exposed to homocysteine than the mean of all homocysteine exposed samples ($P < 0.0001$).

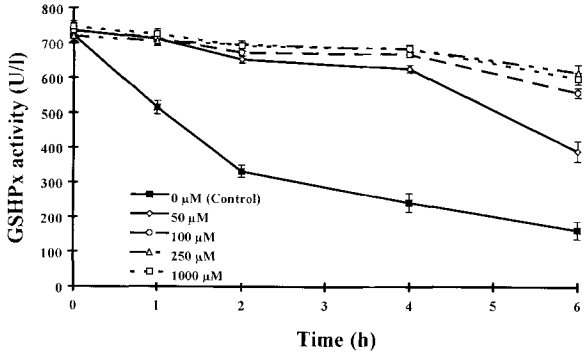

FIGURE 7 Effects on enzyme activity of incubating purified GSHPx with a range of different cysteine concentrations (Each value represents the mean of four samples ± SEM.) The mean fall in activity was greater in samples not exposed to cysteine than the mean of all homocysteine exposed samples ($P < 0.0001$).

Cysteine had a similar protective effect on GSHPx activity over a 6 h incubation (Figure 7). The activity at 6 h was greater in the samples incubated with cysteine than the control sample ($P < 0.001$). These effects were concentration-dependent; samples incubated with 50 μM cysteine showed significantly greater reduction in activity than that at a cysteine concentration of 1000 μM ($P < 0.05$).

DISCUSSION

In this study, an elevation of plasma tHcy was associated with an unequivocal increase in the activity of plasma GSHPx. The same association was observed in a single subject from whom multiple blood samples had been collected over 6 months. An increase in red blood cell SOD activity just failed to reach statistical significance when corrected for the confounding effect of age.

The increase in activity of GSHPx can be explained, at least in part, by an increase in the amount of enzyme in the plasma. When exposed to oxidative stress cells can respond by increasing their antioxidant enzyme activity, which may help avoid injury to the cell. There is limited evidence that the expression of mRNA for GSHPx and SOD may be upregulated in the face of oxidant stress.[25] This group[25] exposed human endothelial cells to oxidative stress imposed by high concentrations of glucose and demonstrated an increase in the activity of and the mRNA expression for SOD, catalase and GSHPx. Similarly, exposure of both endothelial and smooth muscle cells in culture to hyperoxia resulted in the induction of CuZn SOD.[26] In patients with homocystinuria, autoxidation of homocysteine, with the concomitant generation of ROS,[8,10,27] may also induce the upregulation of antioxidant enzymes, including erythrocyte SOD and the plasma isoform of GSHPx, originating in the kidney.[28]

There is very limited information regarding antioxidant activity in response to elevated homocysteine. The only other report of a human study describes a modest increase in the erythrocyte SOD activity in obligate heterozygotes for homocystinuria following a methionine load, compared with normal controls, although this difference did not reach statistical significance. There was no increase in erythrocyte catalase or GSHPx activity.[29] The degree of hyperhomocysteinaemia in these individuals was modest (mean plasma tHcy concentration 50 μM). In an attempt to induce chronic hyperhomocysteinaemia, Toberek and colleagues[30] fed rabbits a methionine-rich diet over 9 months. This treatment elicited an increase in erythrocyte SOD activity but a concomitant fall in erythrocyte GSHPx activity. It is assumed that hyperhomocysteinaemia resulted, although plasma tHcy was not reported.

More recently, Upchurch *et al.*[31] using an endothelial cell culture model, demonstrated that with increasing homocysteine concentrations (50–1000 μM), the activity of the endothelial form of GSHPx decreased in a dose-dependent manner. A similar effect on GSHPx activity was observed by Nishio *et al.*[24] on smooth muscle cells in culture, although in this cell line increasing homocysteine concentration also resulted in an increase in SOD activity.[27] This same group explored the effect of homocysteine on the activity

of purified bovine cytosolic GSHPx and were able to reproduce the effects of elevated homocysteine observed in their cultured cells. In direct contrast to Nishio *et al.*[24] we have demonstrated that homocysteine can protect GSHPx in both plasma and in a purified form (bovine erythrocytes) from loss of activity during prolonged incubation. The fact that another thiol-containing amino acid (cysteine) could provide a similar degree of protection suggests very strongly that *in vitro*, homocysteine simply acts as a reducing agent to stabilise the conformational structure of the enzyme and preserve enzyme activity. This confirms early observations made by Condell and Tappel.[32]

The association between elevated plasma homocysteine and risk of cardiovascular disease has prompted intense speculation as to the likely mechanisms. We describe a significant increase in activity of plasma GSHPx and a trend towards an increased erythrocyte SOD activity in blood samples with elevated plasma homocysteine. For GSHPx the increased activity can, at least in part, be attributed to increased enzyme protein. *In vitro* studies confirmed that this effect was not a direct homocysteine-related chemical enhancement of GSHPx activity.

Both SOD and GSHPx are inducible enzymes in that oxidative stress elicits an increase in enzyme protein.[25,26] Elevated plasma homocysteine, which probably reflects intracellular homocysteine formation,[33] may represent an oxidative stress through autoxidation and the associated generation of hydrogen peroxide, homocysteinyl radicals and other ROS.[8,10,34] Work with cells in culture suggests that sites of production of ROS *in vivo* could include endothelial cells and smooth muscle cells.[35,36] Erythrocytes and extracellular fluids are further possible sites of homocysteine autoxidation. The mechanisms leading to the increase in plasma antioxidant enzyme activity in hyperhomocysteinaemia are not yet understood, but may involve a response to elevated homocysteine concentrations in liver and kidney which are sites of very active homocysteine metabolism but may also be sites of synthesis of plasma GSHPx.[28,37]

In conclusion, elevated plasma homocysteine leads to an increased activity of circulating antioxidant enzymes *in vivo*. This may occur in response to oxidative stress posed by elevated plasma tHcy, and serve to minimise free radical-mediated damage.

Acknowledgements

The authors are grateful to the patients and parents who participated in this study. We would like to acknowledge the financial support of The Children's Appeal, Sheffield Children's Hospital and The Ministry of Agriculture, Fisheries and Food.

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