Phospholipid hydroperoxide cysteine peroxidase activity of human serum albumin

Rachel HURST*, Yongping BAO*, Saxon RIDLEY† and Gary WILLIAMSON*¹

* Department of Biochemistry, Institute of Food Research, Norwich Laboratory, Norwich Research Park, Colney Lane, Norwich NR4 7UA, U.K., and

† Intensive Care Unit, Norfolk and Norwich Hospital, Norwich, Norfolk NR1 3SR, U.K.

Human serum albumin (HSA) reduced the phospholipid hydroperoxide, 1-palmitoyl-2-(13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (PLPC-OOH) to the corresponding hydroxy-derivative with a high apparent affinity ($K_m = 9.23 \pm 0.95 \mu\text{M}$). Removal of bound lipid during purification increased this activity. At physiological concentration, HSA reduced the phospholipid hydroperoxide in the absence of a cofactor. However, in the presence of a cofactor (reductant), the rate of the reaction was increased. All of the major aminothiols in plasma could act as reductants, the best being the most abundant, cysteine ($K_m = 600 \pm 80 \mu\text{M}$). For every nanomole of PLPC-OOH reduced by HSA, 1.26 nmol of cystine was formed, indicating a reaction stoichiometry of 1 mol PLPC-OOH to 2 mol cysteine. We used chemical modification to determine which amino acid residues on HSA were responsible for the activity. Oxidation of thiol group(s) by *N*-ethylmaleimide led to

a reduction in the rate of activity, whereas reduction of thiols by either dithiothreitol or the angiotensin-converting enzyme inhibitor, captopril, increased the activity. Both *N*-ethylmaleimide-modified HSA and dithiothreitol-treated HSA exhibited increased apparent affinities for PLPC-OOH. For a range of preparations of albumin with different modifications, the activity on PLPC-OOH was dependent on the amount of free thiol groups on the albumin (correlation coefficient = 0.91). Patients with lowered albumin concentrations after septic shock showed lowered total plasma thiol concentrations and decreased phospholipid hydroperoxide cysteine peroxidase (PHCPx) activities. These results therefore show for the first time that HSA exhibits PHCPx activity, and that the majority of the activity depends on the presence of reduced thiol group(s) on the albumin.

Key words: bilirubin, captopril, cystine, glutathione, thiol group.

INTRODUCTION

Oxidative stress plays a role in the pathogenesis of many diseases, including cancer, atherosclerosis, cataracts and neurodegenerative disorders [1,2]. During oxidative stress, radical chain-reactions may occur, leading to extensive formation of phospholipid hydroperoxides, which ultimately cause damage to tissues and macromolecules [3,4]. Lipid peroxidation products can also cause DNA damage [5]. Hence, the prevention of lipid peroxidation is an essential process. A wide array of enzymic and non-enzymic antioxidant defences exist, including selenium-dependent phospholipid hydroperoxide glutathione peroxidase (Se-PHGPx), glutathione peroxidase, superoxide dismutase, catalase, ascorbic acid, α -tocopherol, glutathione and β -carotene [6]. Many studies have also indicated an apparent antioxidant role of albumin [7,8–10].

Human serum albumin (HSA) is the major transport protein in blood plasma for a wide range of molecules, including drugs, hormones, metal ions, amino acids (notably cysteine and tryptophan) and fatty acids [11]. It is a relatively small (65 kDa), highly soluble protein consisting of 585 amino acids, with 17 disulphide bridges, one free thiol group (Cys-34) and a single tryptophan (Trp-214). The single thiol group plays an important role in the binding of thiol-containing compounds such as cysteine, glutathione and captopril. Captopril is an angiotensin-converting enzyme inhibitor which is used to treat hypertension [12] and is capable of unblocking Cys-34 [13]. This free thiol residue is highly conserved in all serum albumin molecules and is

located in a crevice in the IA subdomain [11,14]. It has been suggested that Cys-34 is the most reactive thiol group in serum [15]. The Trp-214 residue is conserved in mammalian albumins and has an important structural role in the formation of the IIA binding site which is the site for binding of bilirubin. Trp-214 also participates in an additional hydrophobic packing interaction between the IIA and IIIA interface. IIIA is the binding area for long-chain fatty acids [14], and the binding of long-chain fatty acids has been linked to allosteric activation of Cys-34 in HSA [15].

We have previously found that BSA, when used as a carrier in the incorporation of phospholipid hydroperoxide into mammalian cells, results in a slow reduction of the phospholipid hydroperoxide to phospholipid hydroxide [16]. BSA and HSA have 80% identical amino acids and the important Cys-34 residue is conserved [11], hence, we predicted that HSA may also possess phospholipid hydroperoxide reducing activity.

This is the first report of HSA activity on phospholipid hydroperoxides. One study measured the activity of human plasma towards phospholipid hydroperoxides but not isolated HSA [10], another study reported that apolipoprotein A-1 can reduce a phospholipid hydroperoxide [17]. The reduction of phospholipid hydroperoxides is effectively an 'antioxidant' activity. HSA shows antioxidant activity in some assays, and studies have linked the antioxidant activity of HSA to bound bilirubin [18–20]. The present study was undertaken to determine the phospholipid hydroperoxide cysteine peroxidase (PHCPx) activity of freshly purified HSA and also the mechanism of

Abbreviations used: HSA, human serum albumin; PHCPx, phospholipid hydroperoxide cysteine peroxidase; Se-PHGPx, selenium-dependent phospholipid hydroperoxide glutathione peroxidase; PLPC, 1-palmitoyl-2-linoleoyl-L-3-phosphatidylcholine; PLPC-OOH, 1-palmitoyl-2-(13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine; PLPC-OH, 1-palmitoyl-2-(13-hydroxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine; DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

¹ To whom correspondence should be addressed (e-mail gary.williamson@bbsrc.ac.uk).

its action on 1-palmitoyl-2-(13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (PLPC-OOH).

EXPERIMENTAL

Materials

1-Palmitoyl-2-linoleoyl-L-3-phosphatidylcholine (PLPC), soybean lipoxidase (EC.1.13.11.12, type IV), choline chloride, deoxycholic acid, reduced glutathione (GSH), cysteine, homocysteine, cysteinyl glycine, heparin, Bromocresol Green reagent, Bradford reagent, 1-fluoro-2,4-dinitrobenzene, bilirubin, captopril and the commercial source of HSA were purchased from Sigma Chem. Co. Ltd (Poole, U.K.). HSA solution (ZENALB® 4.5, sample III) was obtained from Bio Products Laboratory (Herts., U.K.). Methanol and acetonitrile, of HPLC grade, were filtered and degassed before use. The Ultrafree-15 centrifugal filter devices (10 kDa cut-off) were manufactured by Millipore (Herts., U.K.). The Ultracarb 5 ODS (20) (250 × 4.6 mm) column was from Phenomenex (Cheshire, U.K.) and the Waters Spherisorb S5 Amino (200 × 4.6 mm) column was from HPLC Technology (Cheshire, U.K.). Fresh blood was obtained from known healthy donors or from patients who had septic shock, at the Norfolk and Norwich Hospital, Norwich. The work was approved by the Norwich District Ethical Committee and carried out in accordance with the Declaration of Helsinki of the World Medical Association. Subjects gave informed consent to the work. All other reagents were of analytical grade and available commercially.

Preparation of plasma

Freshly drawn human blood samples were immediately placed on ice and centrifuged at 1580 *g* at 4 °C for 15 min. The plasma was carefully removed without disturbing the bottom layer of red blood cells and kept on ice (for up to 12 h) until thiol analysis and activity measurements were carried out.

Purification of HSA

HSA was purified from fresh blood obtained from normal donors, with a heparin–Sephacrose CL-6B column and a Cibacron Blue 3GA fast flow matrix [21]. The concentrated and de-salted HSA sample was purified further by removal of hydrophobic ligands, such as steroids, lipids and protein-bound fatty acids, using a Lipidex column [22]. This yielded pure HSA which displayed a single band on SDS/PAGE at 65 kDa and possessed the N-terminal amino acid sequence Asp-Ala-His-Lys-Ser (samples I and II).

Determination of albumin and protein concentration

Albumin concentration was determined using the Bromocresol Green assay [23,24]. Determination of the amount of protein in all samples was carried out using the dye-binding assay of Bradford [25] with a commercial source of HSA as the standard. The absorbance at 280 nm was also measured for the pure albumin samples and the amount of protein was calculated using a molar absorption coefficient of $3.5 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [26].

Determination of free thiol groups

Free thiol groups were measured using a modification of Ellman's method [27]. HSA (10 μl) was incubated with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) solution (1 mg per ml, 100 μl) for 15 min and the absorbance at 410 nm was measured. Saturated

N-ethylmaleimide (NEM) (1 μl) was then added and the absorbance was read again after 10 min. The calculation of free thiol groups was carried out as described [27].

Modification of the thiol group

The thiol group of HSA was modified in a similar procedure to that described by Cha and Kim [7]. HSA was incubated with dithiothreitol (DTT) (2 mM) and NaCl (100 mM) for 20 min at 30 °C, then NEM (10 mM) was added and the mixture was incubated for a further 2 h. Before measurement of activity, excess reagent was removed using an Ultrafree-15 centrifugal filter and the change in thiol group was measured as above. HSA was also treated with captopril (0.5–500 mM) to modify the thiol group.

Preparation and purification of PLPC-OOH

PLPC-OOH was prepared from PLPC using soybean lipoxidase as described by Maiorino et al. [28]. The PLPC-OOH was separated from unoxidized phospholipid by FPLC on a PepRPC HR5/5 column with a water to methanol gradient. The concentration of the phospholipid hydroperoxide collected in 100 % methanol was determined from the absorbance at 232 nm ($\epsilon = 2.5 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) [29]. 1-Palmitoyl-2-(13-hydroxy-*cis*-9,*trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (PLPC-OH) standards were prepared as described by Bao et al. [30].

HPLC analysis of PLPC-OOH and PLPC-OH

HSA activity towards PLPC-OOH was determined using a sensitive and specific HPLC method as described by Bao et al. [30]. The assay mixture contained 0.1 M Tris/HCl (pH 7.4), 2 mM EDTA, 1 mM NaN_3 , 0.25 mM cysteine, 25 μM PLPC-OOH and the appropriate amount of HSA (~0.5 mg) or plasma (10 μl) in a final volume of 0.5 ml. The reaction was carried out at 37 °C for 20 or 30 min and termination was achieved by the addition of ice-cold acetonitrile. Before HPLC analysis the samples were centrifuged at 11 600 *g* for 2 min. An Ultracarb 5 ODS (20) column (250 × 4.6 mm) at 30 °C was used to separate PLPC-OOH and PLPC-OH. The mobile phase consisted of acetonitrile, methanol and water (50/49.5/0.5, by vol.) containing 10 mM choline chloride. The flow rate was maintained at 0.5 ml/min and detection was at 232 nm. Conversion of substrate into product was determined from the peak height of the PLPC-OH standards (see above).

Kinetic studies

The K_m and V_{max} values of HSA for cysteine with PLPC-OOH as the substrate were determined by triplicate measurements of activity at various concentrations of cysteine. PLPC-OOH was added at a fixed concentration (25 μM) and cysteine concentration ranged from 0.01 to 1.0 mM. Kinetic parameters for HSA towards PLPC-OOH were also determined using a fixed concentration of cysteine (0.25 mM) and a range of PLPC-OOH concentrations from 2 to 40 μM . Data analysis of the results was carried out using the method described by Wilkinson [31].

HPLC analysis of cystine

Quantification of the conversion from cysteine into cystine allowed determination of the stoichiometry of the HSA reaction with PLPC-OOH. Dinitrophenol derivatization and HPLC analysis were carried out as described by Fariss and Reed [32]. Briefly, samples were prepared by addition of γ -glutamyl gluta-

mate (15 mM), bathophenanthroline disulphonic acid (15 mM) and 70% perchloric acid. After mixing, freezing and thawing, the samples were centrifuged at 15000 *g* for 3 min. An aliquot of the supernatant was removed for derivatization: iodoacetic acid (100 mM) and KOH (2 M)/KHCO₃ (2.4 M) were added to the aliquot and the mixture was incubated in the dark for 10 min. Finally, 1% 1-fluoro-2,4-dinitrobenzene was added and the samples were left at 4 °C overnight. The derivatized samples were centrifuged (15000 *g* for 3 min) before injection on the Spherisorb S5 Amino column. A gradient system of elution was used with two mobile-phase buffers (A: 80% methanol; and B: 0.5 M sodium acetate in 64% methanol). The flow rate was maintained at 1.5 ml/min and the detector was set at 365 nm. Cystine eluted during a 10 min linear gradient from 20 to 99% B. The cystine from the reaction mixture was quantified relative to external standards of cystine. The response of the detector was linear between 2 and 125 nmol of cystine.

Binding study with bilirubin

Bilirubin was dissolved in 0.05 M sodium hydroxide immediately before it was added to HSA in 10 mM potassium phosphate buffer (pH 7.0). HSA was treated with a wide range of bilirubin concentrations ranging from 10 μ M to 0.5 mM and the effect on HSA activity towards PLPC-OOH was measured. For the control sample with no bilirubin the appropriate amount of sodium hydroxide was added.

Statistical analysis

A Student's *t*-test was used to explore any differences between specific activity and thiol group content after treatment with DTT and/or NEM and also between the plasma samples from normal donors and from patients who had septic shock. A *P* value of < 0.05 was considered significant.

RESULTS AND DISCUSSION

HSA activity towards PLPC-OOH

Figure 1 shows the HPLC measurement of the activity of freshly purified HSA towards PLPC-OOH. Under the specific HPLC conditions employed, the mean retention times of PLPC-OOH and PLPC-OH were 17.6 ± 0.03 and 19.0 ± 0.03 min respectively ($n = 40$). PLPC-OOH was stoichiometrically converted into

Figure 1 HPLC measurement of PLPC-OOH reduction by HSA

PLPC-OOH (25 μ M) was incubated at 37 °C for 30 min with (solid line) or without (dotted line) HSA plus Tris/HCl (0.1 M; pH 7.4), EDTA (2 mM), NaN₃ (1 mM) and cysteine (0.25 mM). The arrows indicate the elution positions of PLPC-OOH and PLPC-OH standards.

Figure 2 Formation of PLPC-OH over time

PLPC-OOH (25 μ M) was incubated at 37 °C with plasma (top) or purified HSA (0.5 mg) with cysteine (0.25 mM) (bottom) in Tris/HCl (0.1 M; pH 7.4), EDTA (2 mM) and NaN₃ (1 mM). Formation of PLPC-OH was quantified by the HPLC method as described in the Experimental section. Data points are means of triplicate determinations and the error bars indicate S.D.

Figure 3 Formation of PLPC-OH as a function of albumin concentration

The standard HPLC assay was used with appropriate amounts of HSA added, see the Experimental section. Data points are the means of at least triplicate determinations and the error bars indicate S.D.

PLPC-OH in the presence of HSA, with or without reductant added. The conversion from PLPC-OOH into PLPC-OH was linear over the time intervals used in the standard assay: 20 and 30 min for plasma and purified HSA respectively (Figure 2). A linear relationship was found between formation of PLPC-OH and HSA concentration up to ~ 2 mg/ml HSA (~ 30 μ M) (Figure 3) and all amounts of HSA used in the standard assay were within the linear range.

Table 1 HSA activity towards PLPC-OOH with a variety of reductants

Reductants were added at the same concentration (0.25 mM). Specific activities are the means of at least triplicate results $\pm$ S.D. Activity was determined at 25 μ M PLPC-OOH with 0.25 mM of the substrate listed at 37 °C, pH 7.4. All specific activities have been adjusted for the control value with no HSA present.

Reductant	Specific activity (nmol/min per mg of protein)
Cysteine	0.07 ± 0.001
Glutathione	0.05 ± 0.004
Cysteinyl glycine	0.04 ± 0.008
Homocysteine	0.02 ± 0.005
None	0.01 ± 0.001

Figure 4 Time course of conversion of PLPC-OOH into PLPC-OH by plasma

PLPC-OOH (25 μ M) was incubated at 37 °C with plasma (61 mg/ml HSA). PLPC-OOH and PLPC-OH were quantified by the HPLC method as described in the Experimental section. Results are the means of duplicate determinations.

The activity of HSA towards PLPC-OOH was measured with the various aminothiols (reductants) present in plasma (Table 1). The greatest activity towards PLPC-OOH was with cysteine, the most abundant aminothiol in plasma [33]. Removal of hydrophobic ligands by a Lipidex column increased this PHCPx activity of HSA by 60%. HSA also reduced the phospholipid

hydroperoxide in the presence of the other aminothiols, but their concentrations in plasma are about 8–25 times lower than that of cysteine [33]. In the absence of reductant, only a small activity of HSA towards PLPC-OOH was observed. Ascorbic acid (0.25 mM) did not alter the activity of HSA towards PLPC-OOH. For the standard HPLC assay, 0.25 mM cysteine was selected together with 25 μ M PLPC-OOH.

Stoichiometry of the HSA reaction with PLPC-OOH and cysteine

Under the conditions of the standard assay, 2.43 ± 0.10 nmol of PLPC-OOH was converted into 2.38 ± 0.11 nmol of PLPC-OH in 30 min ($n = 3$), with concomitant reduction of cysteine to form 3.03 ± 0.15 nmol of cystine (i.e. 1 mol of PLPC-OH formed for 1.27 ± 0.23 mol of cystine) ($n = 3$). This suggests that the stoichiometry for the reaction was: 2 nmol of cysteine converted into 1 nmol of cystine, for every 1 nmol of PLPC-OOH converted into 1 nmol of PLPC-OH. There was reduction of some (23%) of the cysteine by HSA (30 μ M) alone, which was rapid and complete within 3 min. This was allowed to complete before addition of PLPC-OOH for determination of the stoichiometry.

Activity of HSA at physiological concentration

HSA is present in plasma at 35–50 mg/ml in normal subjects [11]. When the activity of HSA at physiological concentration was measured, cysteine was not essential for the HSA reduction of PLPC-OOH to PLPC-OH, although the rate was much lower than in the presence of a reductant. When PLPC-OOH (25 μ M) was added to HSA (51.5 mg/ml), the albumin reduced 4% of the PLPC-OOH in 30 min with no cysteine present. However, with cysteine present, the reduction of PLPC-OOH increased 4-fold. Since cysteine was present at the normal physiological level (0.25 mM) [33], this implies that HSA in plasma could reduce 0.13 μ M of PLPC-OOH per min.

The rate of reduction of PLPC-OOH by plasma ranged from 0.26 μ M/min (initial rate) to 0.14 μ M/min after 3 h incubation (Figure 4). Within 3 h all of the PLPC-OOH (25 μ M) had been reduced by the phospholipid hydroperoxide reducing activity in plasma. It is important to note that at physiological concentrations of HSA, the reaction is saturated with respect to albumin. Other studies have found reduction of PLPC-OOH by plasma within the range determined in the present study [17,20]. Apolipoprotein A-I in plasma showed lower levels of activity on PLPC-

Table 2 Effect of thiol group modification on the activity of HSA towards PLPC-OOH

HSA samples I and II were freshly purified from human blood and sample III is ZENALB® 4.5. *Treatment with DTT (2 mM) and NaCl (100 mM) for 20 min at 30 °C. †Treatment with NEM (10 mM) for a further 2 h at 30 °C after DTT and NaCl incubation. The specific activities are mean values for at least three replicates and the thiol group amounts are the mean values for at least triplicate measurements $\pm$ S.D. All results have been adjusted for the control data with no HSA present.

HSA sample	Treatment		[Free thiol (SH) group] (mol SH/mol HSA)	Specific activity (nmol/min per mg of protein)
	DTT*	NEM†		
I	—	—	0.33 ± 0.006	0.07 ± 0.001
I	+	—	1.7 ± 0.03	0.36 ± 0.03
I	+	+	0.02 ± 0.004	0.05 ± 0.003
II	—	—	0.40 ± 0.006	0.07 ± 0.003
II	+	—	2.0 ± 0.04	0.37 ± 0.01
II	+	+	0.02 ± 0.007	0.06 ± 0.003
III	—	—	0.50 ± 0.005	0.06 ± 0.004
III	+	—	1.60 ± 0.02	0.23 ± 0.02
III	+	+	0.06 ± 0.004	0.04 ± 0.002

Figure 5 Effect of captopril on the PHCPx activity of HSA

HSA was treated with captopril (0.5–500 mM) and PHCPx activity towards PLPC-OOH was measured using HPLC. Error bars indicate S.D. of triplicate measurements.

Figure 6 Relationship between free thiol (sulphydryl) content and PHCPx activity of HSA

PHCPx activity of freshly purified HSA and modified HSA was determined using HPLC and the amount of free thiols was analysed as described in the Experimental section. Error bars indicate S.D. of at least triplicate results. The results suggest a linear association with a correlation coefficient greater than 0.9.

OOH, and the preparation may have been contaminated with an unidentified protein (possibly HSA) at ~ 65 kDa [17]. The results presented in the present paper suggest that the majority of the plasma activity on PLPC-OOH arises from the PHCPx activity of HSA. In addition, when an equal amount of HSA and plasma (amount calculated as mg of albumin protein) were added to the

standard assay conditions, 1.6 nmol of PLPC-OOH was converted into PLPC-OH in both cases.

Effect of modification of the thiol group

The initial free thiol content of various preparations of HSA were measured (Table 2). The values were similar to a previous study [34] and only slightly lower than other reported values [11,35,36]. A commercial source of dried HSA was found to have a significantly lower thiol content (0.02 ± 0.007 mol thiol/mol HSA; $n = 9$) than the freshly purified HSA. After treatment of HSA (samples I, II and III) with DTT and NaCl, an increase in the free thiol content was observed as expected. The number of free thiol groups increased above one, which indicates that one of the disulphide bonds may have been reduced, since albumin only has one free cysteine (Cys-34). The increase in free thiol content was accompanied by a proportional increase in PHCPx activity (Table 2). This trend was also observed when HSA was treated with captopril, which unblocks Cys-34 in albumin; the effect of captopril on PHCPx activity is displayed in Figure 5. After NEM treatment of HSA, the free thiol content decreased to a value close to zero, as expected by blocking thiol groups; there was also a decrease in the PHCPx activity (Table 2). Generally, significant differences were found for both specific activities and free thiol group contents when HSA was treated with DTT and/or NEM or with captopril. An association between the PHCPx activity of HSA and the amount of free thiol groups for various samples is clear, with a correlation coefficient of 0.91 (Figure 6).

Kinetic parameters for DTT- and NEM-modified HSA

The kinetic parameters for unmodified HSA, DTT- and NEM-modified HSA are displayed in Table 3. Michaelis–Menten kinetics were followed for both cysteine and PLPC-OOH as the substrate. This is in contrast with Se-PHGPx and glutathione transferase Alpha 1-1 (GSTA1-1), which are not saturable by peroxides [37]. K_m and V_{max} values with cysteine and PLPC-OOH as substrates decreased to approx. half of the original value when HSA was modified with NEM, which indicates an increase in apparent affinity for the substrates but a reduction in the catalytic rate. DTT modification of HSA also increased the apparent affinity for the substrates, but, unlike NEM modification, resulted in an increase in the catalytic rate.

Effect of bilirubin binding to HSA

We determined whether PHCPx activity was associated with bound bilirubin, since bilirubin binds to the IIA binding site on HSA [14] and has an antioxidant activity [18–20]. A range of

Table 3 Kinetic parameters for cysteine and PLPC-OOH as substrates for fresh, DTT-treated and NEM-modified HSA

Values for K_m and V_{max} are the means of triplicate measurements from two independent experiments $\pm$ S.D. Kinetic parameters for cysteine were determined using a fixed concentration of PLPC-OOH (25 μ M) and various concentrations of cysteine ranging from 0 to 1.0 mM. Kinetic parameters for PLPC-OOH were determined using a fixed concentration of cysteine (0.25 mM) and various concentrations of PLPC-OOH ranging from 0 to 40 μ M. All results have been adjusted for the control data.

HSA	Cysteine		PLPC-OOH	
	K_m (μ M)	V_{max} (nmol/min per mg of protein)	K_m (μ M)	V_{max} (nmol/min per mg of protein)
Fresh unmodified	600 ± 80	0.21 ± 0.02	9.23 ± 0.95	0.11 ± 0.01
DTT treated	60 ± 9	0.45 ± 0.01	5.12 ± 0.85	0.60 ± 0.03
NEM modified	300 ± 30	0.16 ± 0.01	4.24 ± 0.98	0.06 ± 0.01

Figure 7 Relationship between the free thiol (sulphydryl) content and PHCPx activity of plasma from normal and septic shock subjects

PHCPx activity was measured by HPLC and the free thiol groups were analysed as described in the Experimental section. Data points represent the means of triplicate readings of individual subjects: (●) normal plasma ($n = 18$), (■) plasma from septic shock subjects ($n = 15$).

bilirubin concentrations from 10 μ M to 0.5 mM had no effect on the activity of HSA towards PLPC-OOH, which demonstrates that bound bilirubin does not influence PHCPx activity.

PHCPx activity of HSA and importance of the free thiol group, Cys-34

The PHCPx activity of HSA is shown to be dependent on the free thiol content of HSA. The free cysteine residue, Cys-34, is therefore probably responsible for the majority of this activity, especially since captopril, which can specifically unblock Cys-34 [13], increased the PHCPx activity. However, other thiols may be derived after treatment of albumin with DTT, which may also contribute to the activity.

In septic shock, patients experience a loss of albumin from the plasma. We examined plasma from septic shock subjects for free total thiol groups and for PHCPx activity (Figure 7). The results clearly show that the free thiol content of the plasma was significantly lower (P value < 0.05) in septic shock subjects compared with normal subjects. The phospholipid hydroperoxide reducing activity of plasma was also significantly reduced in septic shock (P value < 0.05). Decreased HSA thiol content has also been reported in coronary artery disease [38]. Furthermore, the free thiol content of albumin may be important for blood products such as ZENALB[®] 4.5 which is used for intravascular volume replacement in: (i) hypovolaemic shock associated with blood loss, trauma and surgical procedures; (ii) burn injuries; (iii) therapeutic plasma exchange; and (iv) in the resuspension of recycled blood cells during major surgical procedures, e.g. liver transplant.

Residual activity after oxidation of thiol groups

The free thiol groups are important for activity of HSA towards phospholipid hydroperoxides, but HSA maintains a residual activity of ~ 0.04 nmol $\cdot$ min⁻¹ $\cdot$ mg⁻¹ when this group is oxidized. The activity is albumin dependent, after subtraction of the values obtained from the control reaction with no HSA, but is much lower than unmodified HSA.

Significance of PHCPx activity

In conclusion, the majority of the HSA activity towards PLPC-OOH is due to free thiol group(s) (including Cys-34) on the protein. The relatively low specific activity on PLPC-OOH is compensated for by its high concentration as the major soluble protein constituent of the circulatory system. With an average concentration of ~ 50 mg/ml, and assuming 100% reduction of the thiol group *in vivo*, from the calculations above HSA is capable of reducing ~ 3500 nM of PLPC-OOH per min; the usual concentration of total phosphatidylcholine hydroperoxides in plasma is about 20–430 nM [39,40]. Hence, this PHCPx activity indicates a very significant and important role of HSA in protection against plasma phospholipid peroxidation.

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