Antioxidant role of human haptoglobin

Chi Feng Tseng, Chia Ching Lin, Hsing Yi Huang, Huei Chun Liu and Simon J. T. Mao

Institute of Biochemical Engineering, College of Biological Science and Technology,
National Chiao Tung University, Hsinchu, Taiwan

Human plasma haptoglobin (Hp) is classified according to three phenotypes: Hp 1-1, 2-1, and 2-2 attributed by their two common alleles 1 and 2. Clinically, the 2–2 phenotype is associated with the risk of cardiovascular diseases and diabetes mellitus in patients. In this study, we demonstrate that Hp is an extremely potent antioxidant, which directly protects low density lipoprotein from Cu^{2+}-induced oxidation. Its potency was markedly superior to probucol (one of the most potent antioxidants). Ranking of the IC_{50} of antioxidant activity was as follows: Hp 1-1 > Hp 2-1 > Hp 2-2 > probucol > vitamin E. Blockage of disulfide linkages between Hp subunits, not only abolished the α-helical content but also diminished the ability of Hp to form a complex with hemoglobin. The modified Hp subunits exerted almost 4 times greater antioxidant activity than that of native Hp. To investigate the antioxidant role of Hp on the cellular level, the cDNA of Hp 1-1 was cloned, introduced into the pcDNA3.0 vector which contains the cytomega lovirus promoter and transfected into chinese hamster ovary (CHO)-K1 cells. Following transfection, CHO cells were able to express Hp 1–1 protein and significantly ($p < 0.001$) elevated cell tolerance against oxidative stress. Transfected cells showed 2-fold higher resistance to hydrogen peroxide exposure for 24 h compared to control cells. Thus, Hp plays a provocative antioxidant role as demonstrated by our *in vitro* and *ex vivo* studies.

Keywords: Antioxidant / Haptoglobin cDNA / Haptoglobin phenotypes / Hemoglobin / Physiologic role

Received 16/12/03
Revised 1/2/04
Accepted 11/2/04

1 Introduction

Hemoglobin (Hb) is the most abundant and functionally important protein in erythrocytes. However, once it is released from red blood cells, it becomes highly toxic because of the oxidative nature of iron-containing heme, which participates in the Fenton reaction to produce reactive oxygen species which cause cell injury [1–2]. Human plasma haptoglobin (Hp), a known acute phase protein [3–5], can capture Hb by forming a high affinity Hp-Hb complex [1, 3, 4]. The complex is then metabolized through a receptor-mediated process involving the CD 163 receptor of macrophages [6]. Therefore, Hb binding by Hp is essential for rapid clearance of Hb from plasma [7]. For this reason, Hp plays a crucial role against Hb-induced oxidative stress by a mechanism thought to involve its high-affinity binding with Hb and prevents iron release from Hb. However, it has yet to be shown that Hp itself is an antioxidant molecule.

Correspondence: Simon J. T. Mao, Ph.D., Professor and Dean, Department of Biological Science and Technology, College of Biological Science and Technology, National Chiao Tung University, 75 PO-Ai Street, Hsinchu, Taiwan
E-mail: mao1010@ms7.hinet.net
Fax: +886-3-572-9288

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane)-dihydrochloride; **CHO**, Chinese hamster ovary; **CM**, carboxymethylated; **CMV**, cytomegalovirus; **Hb**, hemoglobin; **Hp**, haptoglobin; **LDL**, low density lipoprotein

The Hp phenotypes 1-1, 2-1, and 2-2 are attributed to two common alleles, 1 and 2, located at chromosome 16q22.1. Structurally, the minimal unit of Hp (β-α-α-β) is joined by disulfide linkages of the two α and two β chains [6, 8, 9]. Fig. 1 shows that the phenotypes all share the same two β chains (each with M_r 40 000 kDa containing 245 amino acids and about 30% carbohydrate). A typical structure of homozygous Hp 1-1 (β-α1-α1-β) is composed of two identical α1 chains (M_r 9000 kDa containing 83 amino acids). Homozygous Hp 2-2 is composed of two identical α2 chains (M_r 16 500 kDa containing 142 amino acids) whereas heterozygous Hp 2-1 contains one α2 and one α1 chain (Fig. 1). Due to an extra thio group in the α-2 chain, Hp 2-1 and 2-2 form large polymers of monomeric, trimeric, tetrameric, pentameric, hexameric, and even larger arrangement through the disulfide-linkages (Fig. 1). Clinically, diabetic patients with the Hp 1-1 phenotype are markedly resistant to the development of diabetic retinopathy, diabetic nephropathy, and cardiovascular disease (CAD) [10–12]. In a prospective study, participants

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

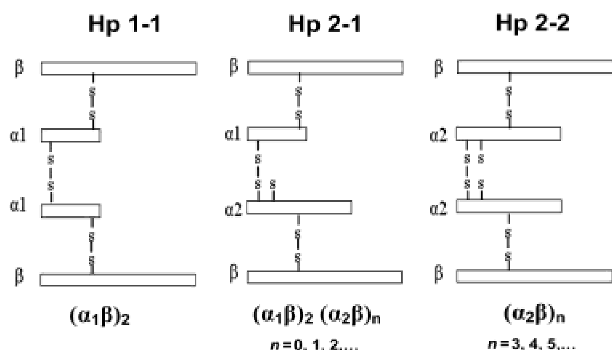

Figure 1. Schematic drawing of the proposed structure of human Hp 1-1, 2-1, and 2-2. All three phenotypes share a common structure of β chains, while Hp 2-1 and 2-2 are heterogeneous polymeric forms. Essentially, the α2 chain represents one entire copy of α1 (1–83) plus an insertion of a partial sequence of α1 (residues 12–70). The degree of polymerization within the intermolecular arrangement is shown. Due to an extra thio group in the β-chain Hp 2-1 and 2-2 can form large polymers (see Fig. 3).

homozygous for the Hp 2-2 allele had a 5-fold increased risk for the development of CAD as compared to participants homozygous for the Hp 1-1, allele [13]. The risk in heterozygous Hp 2-1 participates is intermediate [13].

In the present study, we show that Hp is an extremely potent antioxidant and the activity of Hp 1-1 was differentially and moderately greater than that of Hp 2-1 and 2-2. We also demonstrate that Hp 1-1 transfected Chinese hamster ovary (CHO) K1 cells (which normally do not express Hp) are significantly resistant to oxidative stress. The role of Hp as an antioxidant molecule is therefore identified. The clinical significance with respect to the structure and function of the Hp phenotype is discussed.

2 Materials and methods

2.1 Purification of human Hp

The Hp phenotype was first identified using plasma supplemented with Hb by PAGE, followed by a peroxidase substrate staining [14]. The plasma of each Hp phenotype was then chromatographed on an antibody affinity-column followed by gel filtration chromatography as previously described [14, 15]. The homogeneity of each Hp species employed was greater than 95%.

2.2 Gel electrophoresis

SDS-PAGE containing 15% w/v polyacrylamide gels with a top stacking gel of 5% polyacrylamide was performed for Hp characterization [14, 16]. Samples (typically 5 μg) for SDS-PAGE were preheated at 100°C for 10 min in loading buffer (12 mM Tris-HCl, pH 6.8, 0.4% w/v SDS, 5% v/v glycerol, 0.02% w/v bromphenol blue) with or without 2.88 mM 2-mercaptoethanol. The samples were run for about 1.5 h at 100 V and stained using CBB R-250.

2.3 Western blot

Following protein separation by SDS-PAGE, gels were soaked in a transfer buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.037% w/v SDS, and 20% v/v methanol at pH 8.3. Electrotransfer to a nitrocellulose paper (Pharmacia Diagnostics, Uppsala, Sweden) was performed at 90 mA for 1 h in a semi-dry transfer cell (Bio-Rad, Hercules, CA, USA). Immunoblotting and developing were conducted according to standard procedures previously described [14–16].

2.4 Reduction and carboxymethylation of Hp

Tris-HCl buffer (pH 8.6, 3.3 mL of 0.01 M) containing 5.4 M urea, and 1% v/v β-mercaptoethanol was added to 1 mg HP. The reaction mixture was flushed with nitrogen and incubated at room temperature. After 2 h, 20 mg of iodoacetic acid was added and the reaction mixture was then maintained at pH 8.6 by the addition of 1 M NaOH for 30 min. Finally, carboxymethylated (CM)-Hp was desalted on a Bio-Gel P2 (Bio-Rad, Hercules, CA, USA) column eluted with 0.1 M ammonium bicarbonate and lyophilized [17].

2.5 Preparation of human low density lipoprotein

Human low density lipoprotein (LDL; 1.012–1.063 g/mL) was prepared from human plasma by sequential ultracentrifugation according to the method previously established [17]. Sodium azide (0.01%) was added to the plasma prior to ultracentrifugation and to LDL after isolation. Subsequently, freshly prepared LDL was dialyzed against PBS to remove EDTA and used for oxidation assays [1].

2.6 LDL oxidation

Thiobarbituric acid-reactive substances (TBARS) were used as an index to measure LDL oxidation [1, 18]. In a typical assay, 4 μM $CuSO_4$ and 40 μg of LDL were incubated with native Hp, CM-Hp, probucol, or vitamin E in a final volume of 100 μL. For oxidation induced by water-soluble initiator (5 mM 2,2'-azobis(2-amidinopropane)-dihydrochloride (AAPH)), only Hp 2-1 was employed. Incubation was carried out at 37°C for 2 h, after which

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

time 250 μL of 20% w/v TCA was added to precipitate proteins. Subsequently, 250 μL of 0.67% w/v 2-thiobarbituric acid was added and the reaction was incubated at 80°C for 30 min. The reaction mixtures were centrifuged at 3000 × *g* for 5 min. Supernatant (300 μL) in a 96-well plate was read at 540 nm [1].

2.7 Circular dichroic analysis

Lyophilized Hp was dissolved in 10 mM phosphate buffer (pH 7.4) with a final concentration of 0.2 mg/mL. About 300 μL aliquot was used for the analysis in a cuvette with a 1 mm path length. Circular dichroic spectrum recorded between 190 and 300 nm (in Jasco J-715 spectropolarimetry) was accumulated 20 times at a scanning rate of 50 nm/min. The α-helical content was estimated from the mean residue molar ellipticity (θ_{222}). % α-helix = (θ_{222} + 3000)/(36 000 × 3000) × 100 [17].

2.8 Analysis of Hp-Hb binding complex using HPLC

A HPLC experiment was performed to examine whether or not CM-Hp retains its ability to bind Hb. Hb was purified as previously described [16]. A gel-filtration column (Tosoh, Yamguchi, Japan; TSK-GEL G2000SWXL, 7.8 × 300 mm) pre-equilibrated in PBS was used to analyze the formation of Hp-Hb complex at a flow rate of 0.5 mL/min. A final reaction mixture (100 μL) containing Hp1-1 (24 μg) or CM-Hp 1-1 (24 μg) with and without Hb (16 μg) was subjected to HPLC. Typical chromatographic profiles were monitored at 280 nm, while Hb or Hb-Hp complex formation was monitored at 415 nm.

2.9 Preparation of Hp 1-1 cDNA

Total RNA was extracted from HepG2 cells using TRIzol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's instructions. First strand cDNA was synthesized using 1 μg of total RNA, 30 ng/μL oligo dT, 0.25 mM dNTP, and 3.5 μL diethyl pyrocarbonate water in a 40 μL volume. The mixture was incubated at 65°C for 5 min and chilled on ice. Subsequently, 8 μL 5 × reaction buffer, 4 μL 0.1 M DTT, 2 μL RNase inhibitor (Pharmacia Diagnostics, Uppsala, Sweden) and 1 μL M-MLV RTase (Invitrogen, Carlsbad, CA, USA) were added and incubated at 37°C for 50 min. The reaction was terminated by heating at 70°C for 15 min. PCR was performed in a 50 μL volume containing 0.1 μg of cDNA product, 5 μL 10 × Taq polymerase buffer, 4 μL 2.5 mM dNTP, 2.5 units Taq polymerase, and 50 ng of each specific Hp 1-1 primer (forward: 5'-TCGGTACCATGAGTGCCCTGGAAGCTGTCATTG-3'; reverse: 5'-TCGGTACCTTAGTTCTCAGCTATGGTCTTCTG-3'). The *Kpn I* restriction site is underlined. The thermal cycling program was 40 cycles of 96°C for 30 s, 50°C for 30 s, and 72°C for 1 min. The resulting PCR amplification products were visualized by ethidium bromide on a 1% agarose gel and then recovered using NucleoSpin Extraction Kit (Clontech, CA, USA). The extracted DNA fragment and pcDNA3.0 plasmid (Invitrogen, Carlsbad, CA, USA) were subsequently digested with *Kpn I* at 37°C for 2 h and recovered. Finally, the insert fragment and vector were ligated with a molar ratio of 3:1 at 16°C for 16 h.

2.10 Cell cultures

The CHO-K1 cell line CCL-61 and human hepatoblastoma (HepG2/C3A) CRL-10741 cells obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured in DMEM/F12 (Gibco BRL, Grand Island, NY, USA) and α-minimal essential medium (Gibco), respectively, at 37°C. Media were supplemented with 10% bovine calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin Gibco.

2.11 Immunocytochemistry

Immunostaining was performed using the VECTOR M.O.M. Immunodetection kit (Burlingame, CA, USA) according to the manufacturer's instructions. Briefly, 10^5 CHO cells were cultured in a 6-well plate in which a 76 × 26 mm glass-slide was placed overnight. After washes in PBS, 4% paraformaldehyde was added for 30 min to fix cells. The slide was then immersed in PBS containing 0.1% Tween-20 for 15 min and washed four times with PBS. The slide was hydrogen peroxide and then washed twice immersed in 3% and blocked with M.O.M. mouse Ig blocking reagent for 1 h with two washes. Monoclonal anti-human Hp (1:5000 dilution) was added and incubated for 30 min. Following washes, the slide was incubated with biotinylated anti-mouse IgG for 10 min and washed twice. VECTASTAIN ABC reagent (Vector Laboratories, CA, USA) was added and incubated for 5 min. The slide was washed and developed using 3,3'-diaminobenzidine as a chromogenic substrate.

2.12 Transfection and hydrogen peroxide tolerance assay

The 1044-bp cDNA of Hp 1-1 obtained from human hepatoblastoma HepG2/C3A cells by RT-PCR was cloned into the pcDNA3.0 vector to generate a cytomegalovirus (CMV) promoter-driven Hp 1-1 construct. CHO-K1 cells were transfected with the inserted vector using the

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

SAINT-MIX Gene/Protein-Delivery System Kit (Groningen, The Netherlands), and selected in the presence of 400 μg/mL geneticin. Expression of Hp 1-1 was confirmed by Western blot, immunostaining, and competitive ELISA. Approximately 5×10^3 cells were cultured in a 96-well plate for 24 h and treated with variable amounts of hydrogen peroxide (0–5 mM) for another 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was then used to estimate the relative survival ratio [19].

3 Results

3.1 Antioxidant activity of Hp molecules

To determine Hp antioxidant activity, a method using Cu^{2+}-induced LDL oxidation was employed. Hp was an extremely potent antioxidant against LDL oxidation and acted in a dose-dependent manner (Fig. 2). It was 5 times more potent than probucol [1] and almost 20 times more potent than vitamin E. Notably, the antioxidant activity of monomeric Hp 1-1 was moderately higher than that of polymeric Hp 2-1 and 2-2 (Fig. 2).

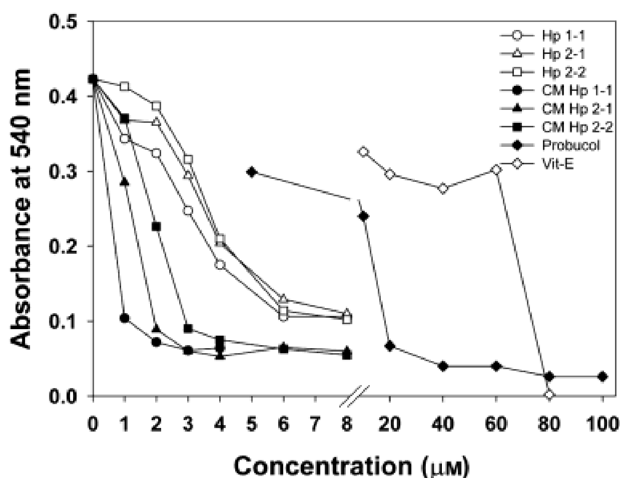

Figure 2. Antioxidant activity of Hp phenotypes. The assay was evaluated using the degree of inhibition of Cu^{2+}-induced formation of thiobarbituric acid-reactive substances from LDL. LDL (40 μg) was incubated with 4 μM Cu^{2+} in the presence of Hp, CM-Hp, probucol, or vitamin E at 37°C for 2 h in a final volume of 100 μL in PBS. BSA (10–100 μM) did not reveal any antioxidant activity (data not shown).

3.2 Effect of disulfide-linkages on the antioxidant property of Hp

Since the disulfide-linkages help maintain the tetrameric arrangement of each Hp monomer, we attempted to address whether or not the overall conformation of Hp plays a key role in its antioxidant function. We found that CM reduction disassembled the α and β chains of Hp on SDS-PAGE without requiring β-mercaptoethanol (Fig. 3). Each chain was identical to Hp subunits following β-mercaptoethanol reduction [14]. Second, using circular dichroic spectrum analysis, we observed a drastic conformational change in Hp following blockage of the disulfide linkage. A representative spectrum is shown in Fig. 4. The estimated α-helical content before CM was about 29, 22, and 21% for Hp 1-1, 2-1, and 2-2, respectively. Statistically, the α-helical content in Hp 1-1 was significantly higher than that in Hp 2-1 and Hp 2-2 ($p < 0.001$). CM-Hp 1-1 had a disordered structure. The conformational change, however, did not attenuate its antioxidant activity. Unexpectedly, it resulted in a marked increase in antioxidant activity (about 4 times; Fig. 2). The ranked antioxidant potency expressed as IC_{50} was: CM-Hp1-1 > CM-Hp 2-1 > CM-Hp 2-2 > Hp 1-1 > Hp 2-1 > Hp 2-2 > probucol > vitamin E (Table 1). BSA did not exhibit any antioxidant activity (data not shown).

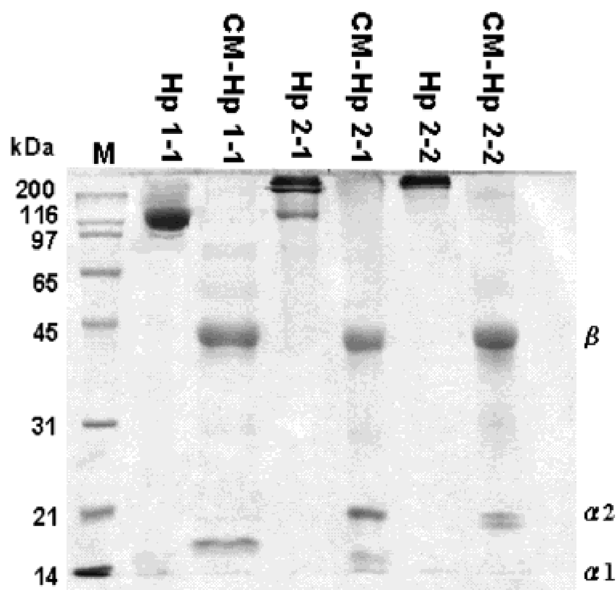

Figure 3. Analysis of CM-Hp on 15% SDS-PAGE without the reducing reagent β-mercaptoethanol. Lane M: protein marker. The α and β subunits are covalently linked in native Hp, but not in CM-Hp 1-1, 2-1, and 2-2 following chemical modification.

3.3 AAPH-induced LDL oxidation

We also tested the ability of Hp to scavenge free radicals generated from a water-soluble azo-compound, AAPH. Hp 2-1 showed dose-dependent antioxidant activity with an IC_{50} about 5 times greater than that of probucol (Table 1).

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

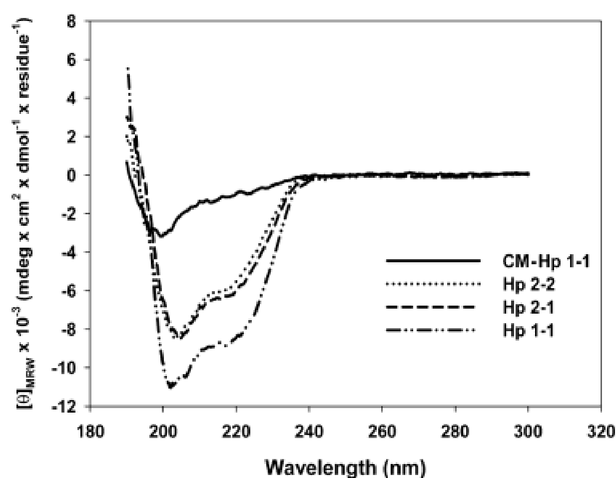

Figure 4. Circular dichroic spectra of native Hp 1-1, 2-1, 2-2, and CM-Hp 1-1. Hp in 10 mM phosphate buffer, pH 7.4, (final concentration 0.2 mg/mL) was monitored by circular dichroism. Each spectrum represents a mean of 20 determinations. The estimated α-helical content is about 29, 22, and 21% for Hp 1-1, 2-1, and 2-2, respectively. The structure of CM-Hp 1-1 is disordered. Statistically, the α-helical content in Hp 1-1 is significantly higher than that of Hp 2-1, 2-2, and CM-Hp 1-1 ($p < 0.001$).

Table 1. Antioxidant activity in Cu^{2+}- and AAPH- induced LDL lipid peroxidation

	Cu^{2+}-induced IC_{50} (μM)[a)]	APH-induced IC_{50} (μM)[a)]
CM-Hp 1-1	0.8	nd
CM-Hp 2-1	1.5	nd
CM-Hp 2-2	2.2	nd
Hp 1-1	3.0	nd
Hp 2-1	3.5	6
Hp 2-2	3.7	nd
Probucol	15	30
Vitamin E	70	nd

a) IC_{50}: the concentration that inhibited 50% of LDL oxidation nd: not determined. See Fig. 2 legend for more details

3.4 Hb-binding ability of CM-Hp

Hp binds Hb forming a high affinity Hp-Hb complex [6]. Presumably, the domain of Hp which binds to Hb is dependent on the overall three-dimensional structure of Hp. We examined the effect of CM of Hp on the formation of the Hp-Hb complex using a HPLC technique. Binding was totally abolished when Hp was CM (Fig. 5). Since the antioxidant activity of CM-Hp was superior to native Hp (Fig. 2), the data indicate that the antioxidant nature of Hp was independent on its binding ability to Hb.

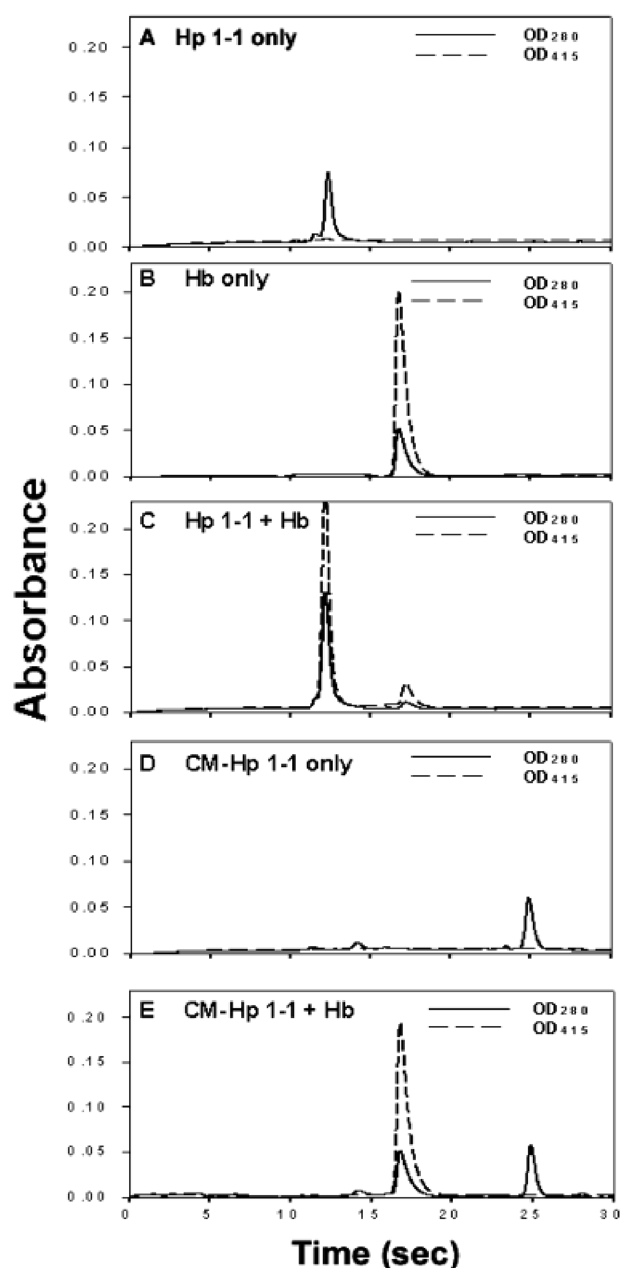

Figure 5. HPLC profile of Hb complexed to native and CM-Hp 1-1. A HPLC gel-filtration column (TSK-GEL G2000SWXL, 7.8 $\times$ 300 mm) was used to analyze the formation of the Hp-Hb complex. A final reaction mixture in 100 μL of PBS containing Hp1-1 (24 μg) or CM-Hp 1-1 (24 μg) with and without Hb (16 μg) was subjected to HPLC. Hb presence was monitored at 415 nm and to 280 nm.

3.5 Expression of Hp 1-1 in CHO-K1 cells

To create an antioxidant cell that may prevent oxidative stress, a CHO-K1 cell line was transfected with a pcDNA3.0 vector containing CMV promoter-driven Hp

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

1-1 cDNA. The plasmid construct also contained a selection marker, geneticin, for conditionally expressing Hp 1-1. Untransfected cells did not express endogenous Hp mRNA as shown by RT-PCR (Fig. 6). Expression of Hp 1-1 protein in the culture medium and CHO cells was confirmed by Western blot analysis (Fig. 7). Only cell lysate from transfected cells had Hp immunoreactivity in a competitive ELISA (data not shown). The specificity of Hp expression in transfected cells was also confirmed by immunocytochemical staining (Fig. 8). The nonimmuned serum did not stain transfected cells (data not shown).

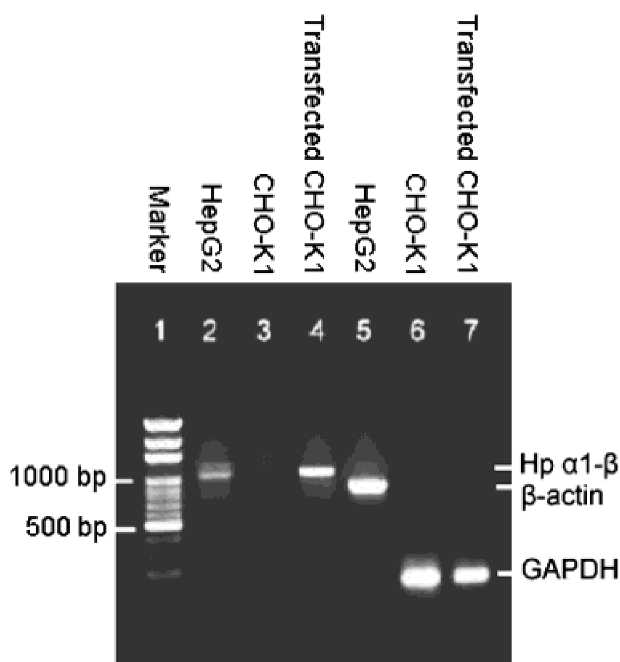

Figure 6. Expression of Hp α1-β mRNA in transfected CHO-K1 cells. PCR was performed to amplify the Hp α1-β cDNA. Lane 1: 100 bp ladder; Lane 2: HepG2 control (Hp α1-β cDNA, 1044 bp); Lane 3: CHO-K1 cells; Lane 4: transfected CHO-K1 cells (Hp α1-β cDNA, 1044 bp); Lane 5: HepG2 internal standard (β-actin, 838 bp); Lanes 6 and 7: CHO-K1 internal standard (GAPDH fragment, 120 bp). The PCR products were analyzed on a 1% agarose gel.

3.6 Activity of Hp transfected CHO cells against oxidative stress

To explore whether the antioxidant cells were resistant to oxidative stress, hydrogen peroxide was added to the CHO-K1 cell culture. Hp 1-1transfected and control cells were treated with variable dosages of hydrogen peroxide for 24 h. As shown in Fig. 9, the relative cell survival ratios of transfected CHO-K1 cells were statistically and significantly higher ($p < 0.001$) than that of untransfected CHO-K1 cells following hydrogen peroxide challenge, especially in the presence of 1 mM H_2O_2. Thus, the expression of Hp elevated the cell tolerance against hydrogen peroxide-induced oxidative stress.

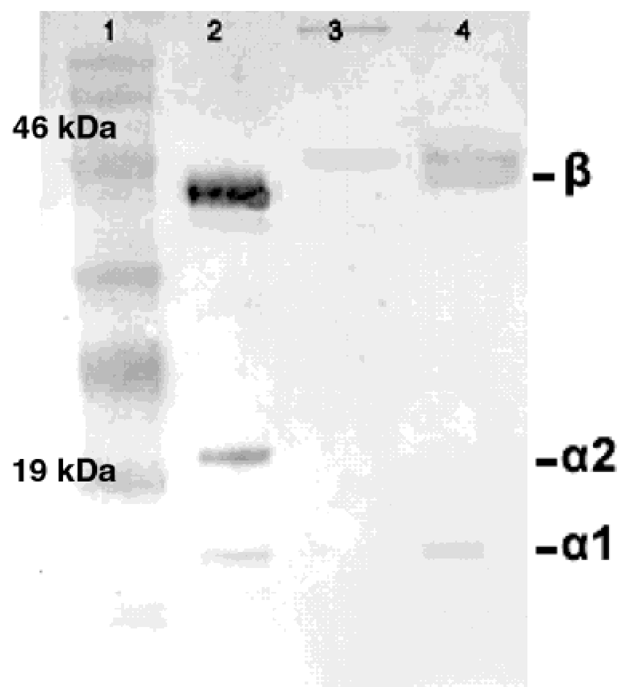

Figure 7. Western blot analysis of Hp expression in culture medium of transfected CHO-K1 cells. CHO-K1 cells were transfected with pcDNA3.0 vector containing CMV promoter-driven Hp α1-β cDNA and cultured in 400 μg/mL of the selection marker geneticin. Transfected CHO-K1 cells exhibited stable expression of Hp over at least 10 passages and more than one month in culture. The collected culture media were concentrated 40-fold, then subjected to 15% SDS-PAGE and Western blot analysis. Lane 1: protein marker; Lane 2: Hp 2-1 standard containing α1, α2, and β subunits; Lane 3: culture media of untransfected CHO-K1 cells; Lane 4: transfected CHO-K1 cells.

4 Discussion

It has recently been demonstrated that Hp can prevent Hb-induced oxidative tissue damage by virtue of its ability to form a high-affinity complex with Hb [7]. Furthermore, Hp 1-1 is superior to Hp 2-1 and 2-2 in binding to Hb [7]. As such, Hp is able to attenuate the release of heme from Hb. In addition to this function, we demonstrate that Hp is also an extremely potent antioxidant molecule in Cu^{2+}- and AAPH-induced LDL oxidation (Fig. 2 and Table 1). The rationale for choosing Cu^{2+}, rather than Fe^{3+}, as a free radical initiator for oxidation was that Cu^{2+} ist similar to Fe^{3+}, forming hydroxyl radicals in the Fenton reaction [1, 20].

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

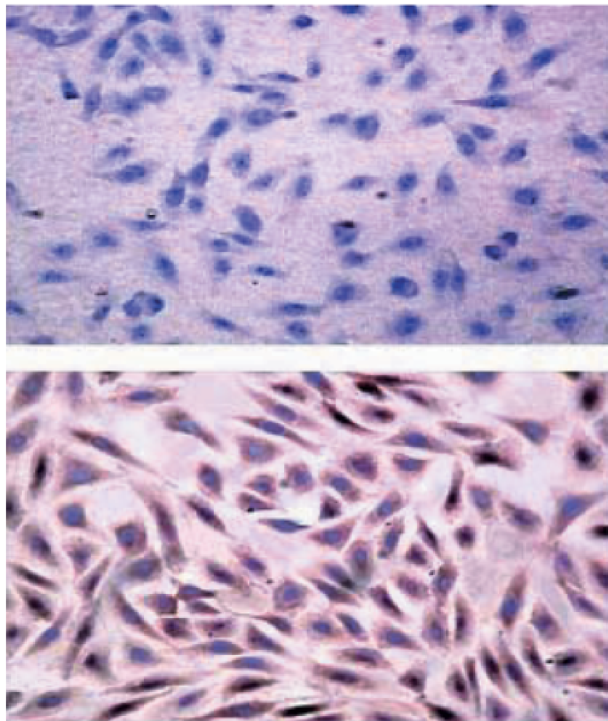

Figure 8. Immunochemical staining for Hp expression in untransfected (top) and transfected (bottom) CHO-K1 cells. 3,3'-Diaminobenzidine was used as the chromogenic substrate for Hp staining and hematoxylin was employed for the nuclear staining. Distribution of Hp (brown) and the nucleus (blue) were observed. The staining was negative when nonimmuned antiserum was used (data not shown).

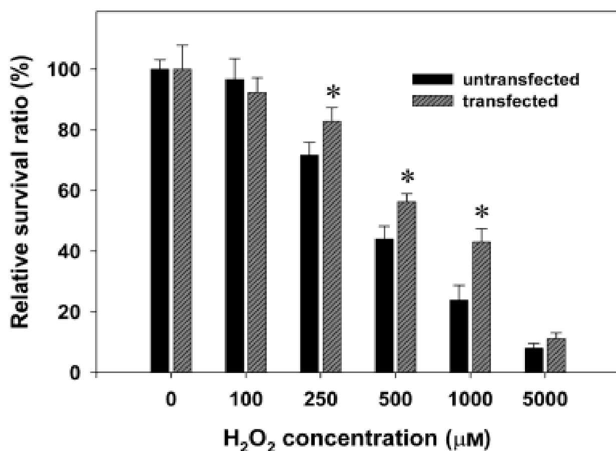

Figure 9. Hydrogen peroxide induced oxidative stress of CHO-K1 cells. Approximately 5×10^3 cells were cultured in a 96-well plate for 24 h, followed by an additional 24 h treatment with variable amounts of hydrogen peroxide (0–5 mM). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was then used to estimate the relative survival ratio. Each bar represents the mean $\pm$ SD in seven determinations. * $p < 0.001$.

Experimental conditions previously reported by us [1, 21–24] were mimicked to compare the antioxidant properties of Hp and probucol. A similar result was seen using Fe^{3+} [25]. Furthermore, a hydrophilic decomposed radical initiator, AAPH, also produced a similar antioxidant effect with Hp 2-1 (Table 1). The antioxidant potency of Hp significantly exceeded probucol [1]. Probucol has been used for the treatment of patients with xanthoma and atherosclerosis for decades [26–30]. Hypothetically, cellular Hp in the cellular level may also play a key role as a natural antioxidant in protection against atherosclerosis.

It is not clear, however, why Hp 1-1 possessed differentially and moderately greater antioxidant activity than Hp 2-1 and 2-2. Evidently, it is not solely due to the polymeric forms of Hp 2-1 and 2-1, since the dissociation of polymeric forms by chemical modification (CN; Fig. 3) did not equalize the antioxidant activity among the phenotypes (Fig. 2). On the contrary, the drastic conformational changes in CM-Hp (Fig. 4) resulted in its 4 times greater antioxidant potency (Fig. 2). We speculate that the region(s) which exert antioxidant activity were further exposed following structural unfolding of Hp. Since the differences in the Hp phenotypes are due to the amino acid sequence of the α-chains, it is essential to analyze the antioxidant domain in this region which may lead to the rational design of new pharmacological agents [1, 21–23]. We are currently in the process of exploring such possibilities.

The diversity in antioxidant activity of the Hp phenotypes may explain, in part, the clinical outcome by which Hp phenotype is associated with differential susceptibility to free-radical related atherosclerosis and autoimmune disorders [5, 31]. A correlation between phenotype-dependent modulation of oxidative stress and prostaglandin synthesis has been reported [31–34]. In general, patients with Hp 2-2 are more susceptible to developing nephropathy in diabetes mellitus. Nakhoul *et al.* [12] postulate that differences in the molecular shape and size of Hp 1-1 and Hp 2-2 may be responsible for the varying susceptibility. Abundant evidence has shown that Hp can be synthesized in the liver, lung, and some fibroblast cells [35]. We have recently reported that it can also be endogenously synthesized in macrophages [36]. At the cellular level Hp can stimulate angiogenin and remodel the arterial wall [37–39]. However, other functions of Hp have not been fully explored. We show that Hp cDNA transfected CHO-K1 cells attain the ability to resist oxidative damage (Fig. 9). This data further substantiates our hypothesis that Hp plays a provocative antioxidant role in the cell.

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

5 Concluding remarks

In conclusion, we demonstrate that Hp is an extremely potent antioxidant molecule and plays a role in cellular resistance to oxidative stress. Blockage of the disulfide linkages of Hp resulted in a loss of its ability to form a complex with Hb. Nevertheless, the modified Hp showed almost 4 times greater antioxidant activity than native Hp. Thus, in addition to its role of blocking the heme group of Hb by forming a complex, Hp may also have an antioxidant role. Whether or not Hp is directly beneficial for free-radical associated atherosclerosis and myocardial infarction [29, 31, 40] will be an interesting and callenging subject for the future.

This work was supported by grant NHRI-EX92-9229SI (S.J.T.M.) from the National Health Research Institute, Taiwan. We also thank the Hsinchu Blood Center of Chinese Blood Services Foundation for kindly providing human plasma.

6 References

[1] Mao, S. J., Yates, M. T., Jackson, R. L., *Methods Enzymol.* 1994, *234*, 505–513.

[2] Tolosano, E., Fagoonee, S., Hirsch, E., Berger, F. G. *et al.*, *Blood* 2002, *100*, 4201–4208.

[3] Raijmakers, M. T. M., Roes, E. M., Morsche, R. H. M., te Steegers, E. A. P., Peters, W. H. M., *J. Med. Genet.* 2003, *40*, 214–216.

[4] Engstrom, G., Stavenow, L., Hedblad, B., Lind, P. *et al.*, *Diabetes* 2003, *52*, 442–447.

[5] Levy, A. P., Hochberg, I., Jablonski, K., Resnick, H. E. *et al.*, *J. Am. Coll. Cardiol.* 2002, *40*, 1984–1990.

[6] Kristiansen, M., Graversen, J. H., Jacobsen, C., Sonne, O. *et al.*, *Nature* 2001, *409*, 198–201.

[7] Melamed-Frank, M., Lache, O., Enav, B. I., Szafranek, T. *et al.*, *Blood* 2001, *98*, 3693–3698.

[8] Maeda, N., Smithies, O., *Annu. Rev. Genet.* 1986, *20*, 81–108.

[9] Patzelt, D., Geserick, G., Schroder, H., *Electrophoresis* 1988, *9*, 393–397.

[10] Levy, A. P., Roguin, A., Hochberg, I., Herer, P. *et al.*, *N. Engl. J. Med.* 2000, *343*, 969–970.

[11] Nakhoul, F. M., Marsh, S., Hochberg, I., Leibu, R. *et al.*, *JAMA* 2000, *284*, 1244–1245.

[12] Nakhoul, F. M., Zoabi, R., Kanter, Y., Zoabi M. *et al.*, *Diabetologia* 2001, *44*, 602–604.

[13] Asleh, R., Marsh, S., Shilkrut, M., Binah, O. *et al.*, *Circ. Res.* 2003, *92*, 1193–1200.

[14] Tseng, C. F., Huang, H. Y., Yang, Y. T., Mao, S. J., *Protein Expr. Purif.* 2003, *33*, 265–273.

[15] Yang, S. J., Mao, S. J., *J. Chromatogr. B, Biomed. Sci. Appl.* 1999, *731*, 395–402.

[16] Liau, C. Y., Chang, T. M., Pan, J. P., Chen, W. L., Mao, S. J., *J. Chromatogr. B, Analyt. Technol. Biomed. Life Sci.* 2003, *790*, 209–216.

[17] Mao, S. J., Sparrow, J. T., Gotto, A. M. Jr., Jackson, R. L., *Biochim. Biophys. Acta* 1980, *617*, 245–253.

[18] Barnhart, R. L., Busch, S. J., Jackson, R. L., *J. Lipid Res.* 1989, *30*, 1703–1710.

[19] Lobner, D., *J. Neurosci. Methods* 2000, *96*, 147–152.

[20] Fenton, H. J. H., *Chem. Soc. J. Lond.* 1894, *65*, 899–910.

[21] Mao, S. J., Yates, M. T., Parker, R. A., Chi, E. M., Jackson, R. L., *Arterioscler. Thromb.* 1991, *11*, 1266–1275.

[22] Dage, R. C., Anderson, B. A., Mao, S. J., Koerner, J. E., *J. Cardiovasc. Pharmacol.* 1991, *17*, 158–165.

[23] Mao, S. J., Yates, M. T., Rechtin, A. E., Jackson, R. L. *et al.*, *J. Med. Chem.* 1991, *34*, 298–302.

[24] Chen, T. H., Tseng, H. P., Yang, J. Y., Mao, S. J., *Life Sci.* 1998, *62*, 277–282.

[25] Thomas, C. E., Jackson, R. L., *J. Pharmacol. Exp. Ther.* 1991, *256*, 1182–1188.

[26] Sia, Y. T., Lapointe, N., Parker, T. G., Tsoporis, J. N. *et al.*, *Circulation* 2002, *105*, 2549–2555.

[27] Roy, K., Saha, A., De, K., Sengupta, C., *Acta Pol. Pharm.* 2002, *59*, 231–234.

[28] Kim, M. H., Cha, K. S., Han, J. Y., Kim, H. J., Kim, J. S., *Catheter. Cardiovasc. Interv.* 2002, *57*, 424–428.

[29] Inazu, A., Koizumi, J., Kajinami, K., Kiyohar, T. *et al.*, *Atherosclerosis* 1999, *145*, 405–413.

[30] Stocker, R., *Curr. Opin. Lipidol.* 1999, *10*, 589–597.

[31] Bernard, D. R., Langlois, M. R., Delanghe, J. R., De Buyzere, M. L., *Eur. J. Clin. Chem. Clin. Biochem.* 1997, *35*, 85–88.

[32] Bernard, D., Christophe, A., Delanghe, J., Langlois, M. *et al.*, *Redox Rep.* 2003, *8*, 41–46.

[33] Langlois, M. R., Delanghe, J. R., *Clin. Chem.* 1996, *42*, 1589–1600.

[34] Frohlander, N., Ljunggren, O., Lerner, U. H., *Biochem. Biophys. Res. Commun.* 1991, *178*, 343–351.

[35] Kalmovarin, N., Friedrichs, W. E., O'Brien, H. V., Linehan, L. A. *et al.*, *Inflammation* 1991, *15*, 369–379.

[36] Mao, S. J., Yang, S. J., Wu, C. H., *FASEB J.* 2001, *15*, A247.

[37] Cid, M. C., Grant, D. S., Hoffman, G. S., Auerbach, R. *et al.*, *J. Clin. Invest.* 1993, *91*, 977–985.

[38] Wassell, J., *Clin. Lab.* 2000, *46*, 547–552.

[39] Cockerill, G. W., Gamble, J. R., Vadas, M. A., *Int. Rev. Cytol.* 1995, *159*, 113–160.

[40] Chapelle, J. P., Albert, A., Smeets, J. P., Heusghem, C., Kulbertus, H. E., *N. Engl. J. Med.* 1982, *307*, 457–463.

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim