Effect of haptoglobin on the metabolism of vitamin C$^{1-3}$

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ABSTRACT  Haptoglobin is a hemoglobin-binding antioxidant showing a genetic polymorphism with three types: Hp 1–1, Hp 2–1, and Hp 2–2. The Hp 2–2 type has been associated with an increased risk of atherosclerosis. We investigated vitamin C metabolism in vivo and in vitro according to haptoglobin type in a study group of 135 healthy volunteers. Serum vitamin C concentrations were associated with haptoglobin type, showing lowest values in serum from Hp 2–2 subjects ($P < 0.01$). Renal threshold for l-ascorbic acid was within the normal range and metabolism to oxalate was not different among haptoglobin-type groups. Serum concentrations of other endogenous antioxidants (uric acid, bilirubin, albumin, ceruloplasmin, and total antioxidative status) were not different among haptoglobin-type groups. In vitro experiments showed a lower stability of l-ascorbic acid in blood from subjects with the Hp 2–2 type ($P < 0.01$). l-Ascorbic acid depletion in vitro was inversely related to haptoglobin concentration ($r = −0.738$). The results of this study indicate a higher rate of l-ascorbic acid oxidation in Hp 2–2 carriers because they have less protection against hemoglobin-iron driven peroxidation. Am J Clin Nutr 1997;66:606–10.

KEY WORDS  Haptoglobin, vitamin C, l-ascorbic acid, antioxidants, hemoglobin-catalyzed oxidation, acute phase protein, humans

INTRODUCTION

Haptoglobin is an acute phase protein with hemoglobin-binding capacity and is characterized by a genetic polymorphism (1–4). Because of the existence of two different alleles (Hp 1 and Hp 2), three main types occur: Hp 1–1, Hp 2–1, and Hp 2–2 (1–4). Haptoglobin is a preventive antioxidant, a consequence of hemoglobin binding to reduce the iron-mediated generation of free radicals (5, 6). After hemolysis, haptoglobin-hemoglobin complexes are formed, which are rapidly eliminated by hepatocytes (7).

Reference serum haptoglobin concentrations are lower in Hp 2–2 subjects (4). The Hp 2–2 type is overrepresented among patients with atherosclerotic diseases (8) and is associated with a higher 5-y mortality rate in patients infected with the human immunodeficiency virus (HIV) (9).

Vitamin C (l-ascorbic acid) is a water-soluble, chain-breaking antioxidant and a powerful scavenger of free radicals (6, 10, 11). Vitamin C is a strong reducing agent and acts as a primary defensive agent in the blood in attack against attack by aqueous radicals (11, 12). Physiologic concentrations of l-ascorbic acid can inhibit low-density-lipoprotein (LDL) oxidation (12–16). Paradoxically, l-ascorbic acid has a prooxidant activity in the presence of Fe$^{3+}$ and promotes hemoglobin-iron driven peroxidation, itself becoming oxidized to l-dehydroascorbic acid (17, 18). Low vitamin C concentrations are associated with an increased risk for atherosclerosis (16). Smokers, patients with diabetes, patients with coronary artery disease, and critically ill patients all have low plasma vitamin C concentrations (16, 19).

We investigated vitamin C metabolism in vivo and in vitro according to haptoglobin type. Serum vitamin C concentrations of subjects with the different haptoglobin types were compared. Measures of other endogenous extracellular antioxidants (uric acid, bilirubin, albumin, ceruloplasmin, and total antioxidative status) were performed simultaneously (6, 20). Because vitamin C is metabolized to oxalate, urinary oxalate excretion was measured as well. Moreover, the rate of l-ascorbic acid depletion in blood samples in vitro was compared among subjects with different haptoglobin types.

SUBJECTS AND METHODS

Subjects

Serum and urine were collected from 135 healthy white volunteers (61 males aged 38 ± 12 y and 74 females aged 40 ± 11 y), after fasting overnight, on a normoenergetic European diet (ie, energy intake between 7.5 and 10.5 MJ, or 1800 and 2500 kcal). Subjects taking vitamin C preparations, vegetarians, pregnant women, smokers, and subjects with a daily alcohol consumption > 20 g were excluded. Subjects had no clinical evidence of malabsorption or other gastrointestinal diseases. All subjects gave informed consent to participate in the study, which was approved by the Ethical Committee of the University Hospital of Gent.

L-Ascorbic acid and total vitamin C assay

Within 30 min after blood samples were taken, l-ascorbic acid concentrations in serum and urine were determined with a Hitachi 911 analyzer by using commercially available reagents (Boehringer, Mannheim, Germany) according to the method described by Beutler (21). In serum, total vitamin C was

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assayed after reduction of the L-dehydroascorbic acid present to L-ascorbic acid by means of dithiothreitol at pH 7.5, followed by measurement of L-ascorbic acid (21). Blood was drawn into small test tubes (4 mL) containing commercial clotting activators (Terumo, Haasrode, Belgium), allowed to clot for 15–20 min, and centrifuged (1000 × g, 10 min, 4 °C). The supernatant serum was immediately put into the analyzer, which was set in emergency testing mode.

**Determination of haptoglobin type and specific proteins**

The haptoglobin type of the subjects was determined by using starch gel electrophoresis of hemoglobin-supplemented serum, followed by peroxidase staining (2). Concentrations of haptoglobin, albumin, ceruloplasmin, and C-reactive protein (CRP) in serum were measured by using fixed-time immunephelometry on a BN II nephelometer (Behringwerke AG, Marburg, Germany), and were expressed according to International Federation of Clinical Chemistry standards (22, 23).

**Measurement of serum and urine indexes**

The urinary oxalate concentration was measured with a commercially available enzymatic method (Sigma Chemical Co, St Louis) on a Hitachi 911 analyzer (Boehringer). The urinary creatinine excretion rate was determined on the same analyzer according to the Jaffé method (24). The ratio of oxalate to creatinine was compared among haptoglobin-type groups. Serum bilirubin and uric acid concentrations were assayed with commercial reagents on a Hitachi 747 analyzer (Boehringer) (24). Total antioxidative status of serum was evaluated with a commercially available spectrophotometric method (Randox, Antrim, United Kingdom) (25).

**Determination of renal threshold for L-ascorbic acid**

After oral ingestion of an aqueous vitamin C solution containing 1.0 g vitamin C of high analytic purity (Fluka Chemie AG, Buchs, Switzerland), renal thresholds for L-ascorbic acid were calculated by linear regression of serum and urinary L-ascorbic acid data. Serum and urine samples were measured in 45-min intervals for 6 h from healthy volunteers (Hp 1–1, n = 4; Hp 2–1, n = 4; and Hp 2–2, n = 4). For any time interval, mean serum and urine L-ascorbic acid concentrations were used for further calculation. The intercept value with the mean serum L-ascorbic acid concentration was considered the renal threshold.

L-Ascorbic acid stability in vitro

The rate of L-ascorbic acid depletion was compared among haptoglobin-type groups. Heparin-treated peripheral blood (20 mL) was collected from four Hp 1–1, four Hp 2–1, and five Hp 2–2 subjects. Total leukocyte counts were determined on an STKS hematometry analyzer (Coulter, Hialeah, FL). To study L-ascorbic acid stability in the absence of haptoglobin, blood was divided into two 10-mL tubes and centrifuged at 1000 × g for 10 min at 4 °C. In one tube, haptoglobin was removed from the supernatant plasma by affinity chromatography with cyanogen bromide–activated hemoglobin-Sepharose 4B (Pharmacia, Uppsala, Sweden). In both tubes, the cell pellets were washed three times with 0.15 mol NaCl/L. The plasma was then resuspended in the cell pellet and vitamin C (Fluka Chemie AG) was added to increase its concentration by 114 μmol/L (20 mg/L). Blood was centrifuged at 1000 × g for 10 min at 4 °C and the initial L-ascorbic acid and haptoglobin concentrations were measured in the supernatant plasma. During incubation at 37 °C for 6 h, plasma L-ascorbic acid concentrations were measured every 2 h.

**Preparation and isolation of haptoglobin-hemoglobin complexes**

Human hemoglobin was prepared from heparin-treated blood. The erythrocytes were washed three times with 0.15 mol NaCl/L, disrupted with 6 mL distilled water, and the nuclei and the ghosts were then removed by centrifugation at 20 000 × g for 20 min. Purified haptoglobin (5 mg) from pooled human plasma (Sigma Chemical Co) was suspended in 1 mL phosphate-buffered saline (Dulbecco A; Unipath, Basingstoke, United Kingdom). Haptoglobin-hemoglobin complexes were obtained by adding 10 mg hemoglobin to 5 mg purified haptoglobin. Consecutively, haptoglobin-hemoglobin and hemoglobin fractions were isolated by means of gel-permeation chromatography with an isotropic HPLC system in phosphate-buffered saline. Therefore, a Waters 650E Advanced Protein Purification System (Millipore, Milford, MA) was combined with a Protein PAK Glass 300 SW column (Waters Nihon Millipore, Tokyo). Concentrations of haptoglobin-hemoglobin and hemoglobin in the collected fractions were estimated by reading the absorbance at 415 nm with a Beckman DU-70 spectrophotometer (Beckman Instruments, Palo Alto, CA). The fractions were adjusted to equimolar concentrations of haptoglobin, haptoglobin-hemoglobin, and hemoglobin. After vitamin C was added to these solutions, the stability of L-ascorbic acid among haptoglobin-type groups was compared as mentioned above.

**Statistics**

All data are given as means ± SDs. Validity of Hardy-Weinberg equilibrium was tested by using the chi-square test. Statistical comparison between haptoglobin-type groups was by one-factor analysis of variance (ANOVA) followed by Student’s t test. Adjustment for multiple testing was done by using the Bonferroni correction. Correlations between data were examined by regression analysis. Statistical significance was set at P < 0.05. MEDCALC software (Mariakerke, Belgium) was used for the analyses.

**RESULTS**

**Haptoglobin gene frequency in the study population**

Haptoglobin-type frequencies were as follows: 0.193 (Hp 1–1), 0.474 (Hp 2–1), and 0.333 (Hp 2–2). Allele frequencies were 0.430 (Hp 1) and 0.570 (Hp 2). These results agree with results obtained by using Hardy-Weinberg’s equilibrium and with results from previous studies of European subjects (4, 8). The age and sex of the subjects according to haptoglobin type are given in Table 1.

**Serum vitamin C and haptoglobin concentrations**

As shown in Table 1, total vitamin C concentrations in serum were significantly lower in Hp 2–2 subjects than in Hp 1–1 and Hp 2–1 subjects. Serum haptoglobin concentrations were similar to those of earlier studies (4, 8) and were lowest in Hp 2–2
subjects. A significant correlation was obtained between serum vitamin C (y, μmol/L) and haptoglobin (x, g/L): y = 6.93x + 56.28 (r = 0.397, SEE = 0.241, P < 0.01).

Endogenous antioxidants and C-reactive protein

Total antioxidant status and serum concentrations of albumin, ceruloplasmin, uric acid, bilirubin, and CRP were not significantly different among haptoglobin-type groups (Table 2). There were no significant correlations between serum vitamin C and endogenous antioxidants or CRP.

Urinary oxalate excretion and renal threshold for l-ascorbic acid

Comparison of the urinary oxalate-creatinine ratio among haptoglobin-type groups showed no significant differences (Table 2). Renal thresholds for l-ascorbic acid were determined after peroral vitamin C ingestion and were 71.1 ± 15.5 μmol/L (Hp 1–1, n = 4), 68.2 ± 14.2 μmol/L (Hp 2–1, n = 4), and 70.3 ± 13.2 μmol/L (Hp 2–2, n = 4). These values correspond to normal renal thresholds for l-ascorbic acid (24). Relative urinary l-ascorbic acid excretion over the 6 h after peroral vitamin C ingestion was 16.8 ± 9.7% (Hp 1–1), 17.0 ± 12.1% (Hp 2–1), and 19.2 ± 8.5% (Hp 2–2).

l-Ascorbic acid stability in vitro

After vitamin C was added to whole blood, the rate of l-ascorbic acid depletion in vitro was compared among haptoglobin-type groups. Initial l-ascorbic acid concentrations in the spiked blood samples were 167.5 ± 8.8 μmol/L (Hp 1–1), 168.2 ± 10.1 μmol/L (Hp 2–1), and 159.4 ± 10.2 μmol/L (Hp 2–2). Total leukocyte counts were not significantly different among haptoglobin-type groups and haptoglobin concentrations were 1.10 ± 0.58 g/L (Hp 1–1), 1.02 ± 0.30 g/L (Hp 2–1), and 0.73 ± 0.31 g/L (Hp 2–2). The percentage decrease in l-ascorbic acid over 6 h was significantly different among haptoglobin-type groups (P < 0.05; Table 3). The rate of l-ascorbic acid depletion correlated with the haptoglobin concentration (Figure 1). After selective removal of haptoglobin from the plasma, l-ascorbic acid stability was not significantly different among haptoglobin-type groups; values were similar to those obtained in Hp 2–2 blood (Table 3).

Effect of hemoglobin on l-ascorbic acid stability

After saturation of purified haptoglobin with hemoglobin, haptoglobin-hemoglobin complexes were separated from hemoglobin by HPLC and vitamin C was added to phosphate-buffered saline solutions containing equimolar concentrations of haptoglobin (0.49 ± 0.11 g/L, n = 3), haptoglobin-hemoglobin (n = 3), and hemoglobin (0.36 ± 0.06 g/L, n = 3). The initial l-ascorbic acid concentration in these solutions was 117.0 ± 10.1 μmol/L. L-Ascorbic acid was more unstable in solutions containing haptoglobin-hemoglobin complexes (47.5 ± 7.3% depletion after 6 h) than in solutions containing equal concentrations of haptoglobin (35.9 ± 5.5% depletion after 6 h). The decay of l-ascorbic acid was even more striking in fractions containing only hemoglobin (62.2 ± 10.8% after 6 h).

DISCUSSION

We showed that serum vitamin C concentrations are influenced by the haptoglobin type. The lowest vitamin C concentrations were found in serum from Hp 2–2 individuals, whereas no significant differences were obtained between the Hp 1–1 and Hp 2–2 groups.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Hp 1–1 (n = 12 M, 14 F)</th>
<th>Hp 2–1 (n = 29 M, 35 F)</th>
<th>Hp 2–2 (n = 20 M, 25 F)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>38 ± 15</td>
<td>39 ± 11</td>
<td>39 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>Vitamin C (μmol/L)</td>
<td>61.5 ± 16.5³</td>
<td>63.7 ± 10.8⁴</td>
<td>49.9 ± 8.5</td>
<td>0.011</td>
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<tr>
<td>Haptoglobin (g/L)</td>
<td>1.26 ± 0.43⁵</td>
<td>1.08 ± 0.50⁴</td>
<td>0.84 ± 0.42</td>
<td>0.005</td>
</tr>
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</table>

¹ ± SD.
² One-way ANOVA of comparison among the three groups.
³, ⁴ Significantly different from Hp 2–2 (post hoc t test): ³ P < 0.01, ⁴ P < 0.005, ⁵ P < 0.001.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Hp 1–1 (n = 26)</th>
<th>Hp 2–1 (n = 64)</th>
<th>Hp 2–2 (n = 45)</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>Albumin (g/L)</td>
<td>41.3 ± 6.5</td>
<td>41.1 ± 5.1</td>
<td>39.6 ± 6.1</td>
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<tr>
<td>Ceruloplasmin (g/L)</td>
<td>0.36 ± 0.11</td>
<td>0.32 ± 0.12</td>
<td>0.33 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Uric acid (mmol/L)</td>
<td>0.31 ± 0.11</td>
<td>0.30 ± 0.08</td>
<td>0.30 ± 0.09</td>
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<tr>
<td>Bilirubin (mmol/L)</td>
<td>7.01 ± 4.45</td>
<td>8.55 ± 5.30</td>
<td>7.87 ± 4.62</td>
<td></td>
</tr>
<tr>
<td>TAS (mmol/L)</td>
<td>1.66 ± 0.16</td>
<td>1.55 ± 0.18</td>
<td>1.69 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>3.1 ± 2.8</td>
<td>2.9 ± 3.5</td>
<td>3.0 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Oxalate:creatinine (%)</td>
<td>2.5 ± 1.1</td>
<td>2.7 ± 0.98</td>
<td>2.2 ± 1.23</td>
<td></td>
</tr>
</tbody>
</table>

¹ ± SD.
² One-way ANOVA of comparison among groups.
³, ⁴ Significantly different from Hp 1–1 and Hp 2–1 (post hoc t test): ³ P < 0.01, ⁴ P < 0.001.

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Hp 1–1 (n = 4)</th>
<th>Hp 2–1 (n = 4)</th>
<th>Hp 2–2 (n = 5)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>% loss over 6 h</td>
<td></td>
<td></td>
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</tbody>
</table>

Plasma with haptoglobin | 24.7 ± 2.5 | 25.9 ± 1.6 | 29.1 ± 2.2³ | 0.023 |
Plasma without haptoglobin | 27.8 ± 1.9 | 28.0 ± 1.5 | 29.5 ± 1.8 | NS |
Difference | 3.1 ± 0.7 | 2.1 ± 0.8 | 0.4 ± 0.3⁴ | 0.005 |

¹ ± SD.
² One-way ANOVA of comparison among groups.
³, ⁴ Significantly different from Hp 1–1 and Hp 2–1 (post hoc t test): ³ P < 0.01, ⁴ P < 0.001.
FIGURE 1. Relation between L-ascorbic acid depletion (y, % loss over 6 h) and haptoglobin concentration (x, g/L) in blood samples from four Hp 1–1, four Hp 2–1, and five Hp 2–2 subjects to which vitamin C was added (114 μmol/L; y = −5.16x + 31.82 (r = −0.738, P < 0.01, SEE = 2.11).

Serum haptoglobin concentrations corresponded to the normal reference values for whites and were lowest in Hp 2–2 individuals (4, 8). A positive relation between serum haptoglobin and total vitamin C concentration was observed. This suggests that degradation of the vitamin decreases with increasing haptoglobin concentration and supports the view that haptoglobin protects against oxidative damage (5, 6).

Theoretically, the low vitamin C concentrations in serum from subjects with the Hp 2–2 type could be caused by three mechanisms: redistribution, oxidation, or renal elimination of the vitamin. The renal threshold for L-ascorbic acid and metabolism rate to oxalate were not significantly different among the three haptoglobin-type groups. Urinary excretion of L-ascorbic acid was not significantly different after peroral loading with vitamin C, suggesting that quantitative L-ascorbic acid uptake by the tissues is not significantly different among haptoglobin-type groups. Therefore, redistribution and elimination are unlikely to be the cause. Our observations can be explained by a higher oxidation of L-ascorbic acid in plasma from Hp 2–2 subjects. Hp 2–2 subjects have haptoglobin concentrations lower than those of Hp 1–1 and Hp 2–1 subjects and are less efficient in removing free haptoglobin from the plasma (4), thereby favoring hemoglobin-iron driven peroxidation.

In contrast with L-ascorbic acid, no haptoglobin-type–dependent significant differences were found for other endogenous reducing agents, such as uric acid, bilirubin, albumin, and ceruloplasmin or total antioxidative status. These results support the view that L-ascorbic acid acts as a primary defensive agent against free radical attack (11, 12). In pathologic conditions, L-ascorbic acid is much more effective than the other water-soluble plasma antioxidants (uric acid, bilirubin, etc) (11, 12). Once L-ascorbic acid has been consumed completely, the remaining antioxidant pools start to decrease (11, 12). Serum albumin and ceruloplasmin concentrations, however, have been reported to be haptoglobin type–dependent (3), but we could not confirm this observation. Furthermore, these findings also suggest that the low serum vitamin C concentrations in the Hp 2–2 group were due to iron-related oxidation of the vitamin because iron-mediated degradation of L-ascorbic acid is especially facile. Although vitamin C and CRP concentrations have been shown to be inversely related in critically ill patients (19), we found no such relation in healthy subjects.

The in vitro experiments showed a higher rate of L-ascorbic acid depletion in blood from Hp 2–2 subjects than in the other haptoglobin-type groups. This suggests that the vitamin is more stable in blood samples taken from Hp 1–1 and 2–1 subjects. The stability of L-ascorbic acid increased with increasing haptoglobin concentration. The protective effect of Hp 2–2 and Hp 1–1 was eliminated by selective removal of haptoglobin from the plasma by affinity chromatography. Saturation of haptoglobin with hemoglobin markedly increased the rate of L-ascorbic acid depletion. In the presence of hemoglobin, L-ascorbic acid was more unstable than in solutions containing haptoglobin-hemoglobin complexes. These in vitro experiments suggest a protective effect of haptoglobin against hemoglobin-iron driven oxidative stress.

Our results indicate that in subjects consuming a vitamin C–poor diet, those with the Hp 2–2 type are most likely to develop vitamin C deficiency. Because significant adverse health effects of moderate vitamin C supplementation in humans have not been reported, it is reasonable to consider vitamin C supplementation in Hp 2–2 patients undergoing oxidative stress (26, 27).

The Hp 2–2 type has been associated with a high risk of atherosclerotic complications (4). In patients with essential hypertension, target organ damage (prevalence of coronary artery disease and peripheral arterial occlusive disease) is more pronounced in Hp 2–2 subjects with hypertension (8). Patients who are candidates for coronary artery bypass grafting who have an Hp 2–2 type develop more severe coronary lesions than do patients with an Hp 1–1 or Hp 2–1 type and have a higher prevalence of acute myocardial infarction in their medical history than other haptoglobin-type groups (28). Oxidatively modified LDL is hypothesized to play an important role in atherogenesis (16). Because dietary vitamin C supplementation has been shown to prevent LDL oxidation (16), we suggest that it be recommended in Hp 2–2 patients with cardiovascular risk factors. Further studies will be required to establish the recommended dietary allowance of vitamin C according to haptoglobin type, apart from the well-known differences according to age, sex, physiologic status (eg, pregnancy), and environmental factors such as smoking, pollution, and alcohol intake.

Further clinical investigations of the potential relation between haptoglobin polymorphism and vitamin C are also needed in HIV-infected patients. The Hp 2–2 type has been associated with a low 5-y survival in patients infected with HIV (9). Oxidative stress induced by reactive oxygen radicals stimulates HIV replication through the activation of the nuclear transcription factor κB, and contributes to the development of tissue damage and immunodeficiency (29, 30). On the other hand, L-ascorbic acid suppresses HIV reverse transcriptase activity and viral replication in chronically HIV-infected cells (31, 32).

In conclusion, serum vitamin C concentrations are lower in Hp 2–2 subjects than in Hp 1–1 and Hp 2–1 subjects apparently because of increased hemoglobin-iron related oxidation of L-ascorbic acid. Further studies are required to establish the requirement and the benefit of vitamin C supplementation on the basis of haptoglobin type.

REFERENCES


