Haptoglobin Genotype– and Diabetes-Dependent Differences in Iron-Mediated Oxidative Stress In Vitro and In Vivo

Rabea Asleh,* Julia Guetta,* Shiri Kalet-Litman, Rachel Miller-Lotan, Andrew P. Levy

Abstract—We have recently demonstrated in multiple independentpopulation-based longitudinal and cross sectional analyses that the haptoglobin 2-2 genotype is associated with an increased risk for diabetic cardiovascular disease. The chief function of haptoglobin (Hp) is to bind to hemoglobin and thereby prevent hemoglobin-induced oxidative tissue damage. This antioxidant function of haptoglobin is mediated in part by the ability of haptoglobin to prevent the release of iron from hemoglobin on its binding. We hypothesized that there may be diabetes- and haptoglobin genotype–dependent differences in the amount of catalytically active redox active iron derived from hemoglobin. We tested this hypothesis using several complementary approaches both in vitro and in vivo. First, measuring redox active iron associated with haptoglobin-hemoglobin complexes in vitro, we demonstrate a marked increase in redox active iron associated with Hp 2-2-glycohemoglobin complexes. Second, we demonstrate increased oxidative stress in tissue culture cells exposed to haptoglobin 2-2-hemoglobin complexes as opposed to haptoglobin 1-1-hemoglobin complexes, which is inhibitable by desferrioxamine by either a chelation or reduction mechanism. Third, we demonstrate marked diabetes-dependent differences in the amount of redox active iron present in the plasma of mice genetically modified expressing the Hp 2 allele as compared with the Hp 1 allele. Taken together these data implicate redox active iron in the increased susceptibility of individuals with the Hp 2 allele to diabetic vascular disease. (Circ Res. 2005;96:435-441.)

Key Words: diabetes ■ oxidative stress ■ iron ■ haptoglobin ■ hemoglobin

The haptoglobin (Hp) gene locus on chromosome 16q22 is polymorphic with two common alleles existing in man, denoted 1 and 2.1 We have recently established in multiple independent population-based longitudinal and cross sectional studies that the haptoglobin genotype is an independent risk factor for diabetic cardiovascular disease.2–6 Specifically, we have demonstrated that diabetic individuals homozygous for the haptoglobin 2 allele (Hp 2-2) are at significantly greater risk of developing cardiovascular disease as compared with diabetic individuals homozygous for the haptoglobin 1 allele (Hp 1-1) with an intermediate risk being found in the heterozygote.3

Haptoglobin is an antioxidant as a direct result of its ability to prevent hemoglobin-driven oxidation.7 The stoichiometric binding of haptoglobin to hemoglobin not only stabilizes the heme iron moiety in hemoglobin (Hb)8 but also promotes its scavenging by the CD163 macrophage receptor by receptor-mediated endocytosis.9 We have recently demonstrated that the ability of haptoglobin to protect against hemoglobin-driven oxidative injury is abrogated when hemoglobin becomes glycated, a process that is markedly accelerated in the diabetic state.10 Glycohemoglobin-haptoglobin complexes are catalytically redox active and therefore the rate at which haptoglobin-hemoglobin complexes are cleared from the serum and extravascular space is of heightened importance in the diabetic state. We have shown that Hp 1-1-Hb complexes are cleared much more rapidly in vitro than Hp 2-2-Hb complexes providing one mechanism for decreased oxidative stress and cardiovascular disease in Hp 1-1 diabetics.10

More than 75% of total body iron is found in hemoglobin, and therefore, genetically determined differences in the disposal and metabolism of hemoglobin-derived iron might be expected to have a profound effect on iron metabolism and iron induced oxidative potential. Although a hypothesis proposing a linkage between total body iron status and atherosclerotic CVD was proposed more than 20 years ago,11 epidemiological studies attempting to demonstrate this link using ferritin or total serum iron as markers of iron status have been generally negative.12–14 However, total body iron may not be reflective of the risk of iron-mediated oxidative injury. Because of the hazardous nature of free iron, elaborate defense mechanisms have been developed so that more than 99% of the iron in the body is sequestered in specialized cells (hemoglobin in red cells), transport or storage proteins (transferrin or ferritin) in which iron is not redox active (ie, incapable of generating hydroxyl radical).15,16 A causal linkage between iron and susceptibility to atherosclerosis is therefore more likely to be related to the amount of iron...
available to catalyze and participate in free radical reactions (redox active iron) rather than total body iron status per se.\textsuperscript{17–22} The molecular nature of such catalytically redox active iron may exist in many forms such as the following: iron bound to albumin or citrate as in thalassemic patients in whom the transferrin binding capacity is exceeded\textsuperscript{21}; iron bound to aberrantly glycated proteins which have an increased affinity for iron\textsuperscript{24}; or hemoglobin-derived iron delocalized outside of the hydrophobic heme pocket as occurs when hemoglobin becomes oxidized and heme iron is oxidized.\textsuperscript{25–29} We hypothesized that there may be diabetes- and haptoglobin type–dependent differences in the amount of catalytically redox active iron derived from hemoglobin. In this article, we have tested this hypothesis at multiple levels demonstrating an increase in catalytically redox active iron release from glycohemoglobin-Hp 2-2 complexes in vitro and increased catalytically redox active iron in the serum and tissues of Hp 2 transgenic mice.

Materials and Methods

Chemicals and Reagents

Reagents were from Sigma Israel (Rehovot) unless specified. Haptoglobin was purified by affinity chromatography.\textsuperscript{10} Glycation of hemoglobin was performed using glycoaldehyde\textsuperscript{30} as described in the expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org.

Measurement of the Affinity of Hemoglobin for Haptoglobin

The affinity of normal and glycohemoglobin for haptoglobin was measured by surface-plasmon resonance (SPR) analysis using the BIACore instrument\textsuperscript{11} and as described in the online data supplement.

Measurement of Redox Active Chelatable Iron

We have used the method of Cabantchik et al\textsuperscript{23} to measure the amount of redox active chelatable iron in a given solution or in plasma. Briefly, dihydrorhodamine (DHR) was used as a sensitive fluorescent indicator of oxidative activity. In the assay to measure redox active chelatable iron, each sample was tested under two different conditions: with 40 μmol/L ascorbate alone and with 40 μmol/L ascorbate in the presence of 50 μmol/L iron chelator (deferiprone). The difference in the rate of oxidation of DHR in the presence and absence of the chelator represents the component of iron that is catalytically redox active. A description of the assay is described in the online data supplement.

Oxidative Stress in Tissue Culture Cells Exposed to Haptoglobin-Hemoglobin Complexes

Intracellular oxidative stress was assessed in CHO cells loaded with DCFH (2,7-dichlorofluorescein) by FACS analysis.\textsuperscript{32,33} CHO cells were incubated with Hp-Hb complexes (100 ng/mL) under conditions of low (100 mg/dL) or high (450 mg/mL) glucose DMEM for 16 hours in the presence or absence of the iron chelator desferrioxamine (DFO) at 10 μmol/L. Results reflect the percentage of cells showing fluorescence attributable to DCFH and are expressed as the percentage change relative to control CHO cells (fluorescence obtained in the CHO cells in the absence of Hp-Hb complex added to the cells with high or low glucose and with or without DFO).

The measurement of lipid peroxides\textsuperscript{34} in the CHO cells is described in the online data supplement.

Association of Iron and Hp in Transgenic Mice

All procedures were approved by the Animal Care Committee of the Technion. All mice were in a C57Bl/6 genetic background. The endogenous wild type murine Hp allele is a type 1 allele.\textsuperscript{35} Mice lacking haptoglobin (Hp knockout or Hp 0) or Hp knockout mice containing a human Hp 2 allele transgene (referred herein as Hp 2 mice) have been previously described.\textsuperscript{36,37} Serum was stored at −20°C. Tissue (kidney) iron was assessed using Prussian Blue.\textsuperscript{38} Diabetes was produced by intraperitoneal injection at 6 weeks of age with streptozotocin at a concentration of 200 mg/kg dissolved in 50 mmol/L citrate buffer pH 4.5. Glucose levels were monitored with a glucometer and a diagnostic kit from Sigma was used to measure HbA1c.

Results

The Affinity of Haptoglobin for Hemoglobin

One potential explanation for why there may be haptoglobin type–dependent differences in oxidative stress is attributable to different affinities between the haptoglobin types for hemoglobin. Moreover, this interaction may be disturbed by glycation of hemoglobin (or haptoglobin). Conventional methods that have been used to assess the interaction between hemoglobin and haptoglobin are not believed to be correct owing to the extraordinary high affinity of haptoglobin for hemoglobin (reported in the literature as kDa of 10\textsuperscript{−10} to 10\textsuperscript{−16} mol/L).\textsuperscript{39} We measured the affinity of this interaction by surface plasmon resonance using the Biacore apparatus, the currently accepted gold standard for measuring the affinity of a biomolecular interaction.\textsuperscript{41} We found that the equilibrium dissociation constant kDa of haptoglobin for hemoglobin was approximately 10\textsuperscript{−18} mol/L and that there was no significant difference between Hp 1-1 and Hp 2-2 (Figure 1 and Table 1). Glycation of hemoglobin in vitro resulted in an approximately 3-fold decline in this affinity for both Hp 1-1 and Hp 2-2. These results indicate that the inability of haptoglobin to prevent oxidation by glycohemoglobin cannot be explained by a change in the affinity of glycohemoglobin for haptoglobin as the kDa of haptoglobin for glycohemoglobin remains several orders of magnitude lower than the concentration of haptoglobin used in vitro and in vivo studies.

Hp 2-2 Fails to Stabilize Hemoglobin-Derived Iron When Hemoglobin Is Glycated

Haptoglobin is thought to inhibit hemoglobin-induced oxidation by preventing the release of redox active iron from hemoglobin.\textsuperscript{7} We sought to determine whether Hp 1-1 and Hp 2-2 differed in their ability to prevent the delocalization of iron from the heme pocket of hemoglobin into a chelatable form that is redox active, and if these differences were affected by the glycation of hemoglobin. Redox active chelatable iron was measured using a kinetic fluorometric assay with dihydrorhodamine (DHR) in the presence of an iron chelator.\textsuperscript{23} A representative assay used to quantify chelatable redox active iron is shown in Figure 2. We found a marked increase in the amount of chelatable redox active iron associated with Hp 2-2-Hb complexes as compared with Hp 1-1-Hb complexes. The differences between Hp 1-1-Hb and Hp 2-2-Hb complexes in the amount of redox active chelatable iron were enhanced when using in vitro glycated Hb (Figure 3).
Increased Oxidative Stress in Cells Incubated With Hp 2-2-Hb Under Hyperglycemic Conditions Is Mediated by Chelatable Iron

We next tried to model haptoglobin type– and diabetes-dependent differences in oxidative stress between Hp 1-1 and Hp 2-2 in a cell-based system by studying the effects of glucose on intracellular oxidative stress produced by Hp 1-1 or Hp 2-2-hemoglobin complexes. We measured intracellular oxidative stress using either a cytofluorimetric assay for intracellular peroxides with DCFH32,33 or by directly measuring Hp 2-2-hemoglobin complexes. We measured intracellular glucose on intracellular oxidative stress produced by Hp 1-1 and Hp 2-2 in a cell-based system by studying the effects of diabetes-dependent differences in oxidative stress between Hp 1-1 and Hp 2-2. We next tried to model haptoglobin type– and diabetes-dependent differences in oxidative stress between Hp 1-1 and Hp 2-2 in a cell-based system by studying the effects of diabetes-dependent differences in oxidative stress between Hp 1-1 and Hp 2-2.

Chelatable Redox Active Iron Is Markedly Elevated in the Plasma of Hp 0 and Hp 2 Mice and Is Further Elevated by Diabetes Melitus

To extend these findings in cell culture to an in vivo setting, we measured chelatable redox active iron in the plasma of diabetic and nondiabetic mice genetically modified at the Hp locus. Six-week-old wild-type (Hp 1-1) mice, Hp 0 mice, and Hp 2 mice all in a C57Bl/6 background were made diabetic using streptozotocin (STZ), and plasma was harvested 40 days after the onset of diabetes mellitus (DM). Glucose and HbA1c were not significantly different between diabetic mice with the different Hp types. We found striking Hp type– and DM-dependent differences in the amount of chelatable redox active iron in the plasma of these mice (Figure 5). In the absence of DM, we found that only 7% of the Hp 1 (wild-type) mice had measurable chelatable redox active iron and that this was not significantly altered by the presence of DM (mean concentration of chelatable iron 5.9±2.0 versus, 6.9±3.6 in the presence or absence of DM; P=NS). However, in Hp 0 (knockout) mice 40% and in Hp 2 mice 30% of the mice had measurable amounts of chelatable redox active iron (17.7±12.1 and 16.8±8.0 mmol/L, respectively). In both the Hp 0 and the Hp 2 mice, this iron component was markedly increased with DM, so that 80% of the Hp 0 and 70% of the Hp 2 mice had elevated chelatable redox active iron in their plasma and the mean concentration of this iron component increased by 2- to 4-fold in the Hp 0 and Hp 2 mice.

**Table 1. Affinity of Haptoglobin for Hemoglobin (± Glycosylation) as Assessed by Biacore**

<table>
<thead>
<tr>
<th>Haptoglobin</th>
<th>Hemoglobin</th>
<th>(K_d)±SD*</th>
<th>(K_d)±SD*</th>
<th>KD (mmol/L)±SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp 1-1</td>
<td>Normal</td>
<td>1.23E+5±0.17E+5</td>
<td>7.70E-5±0.39E-5</td>
<td>0.63±0.09</td>
</tr>
<tr>
<td>Hp 1-1</td>
<td>Glycosylated</td>
<td>4.27E+4±0.16E+4</td>
<td>1.06E-4±0.23E-4</td>
<td>2.47±0.44</td>
</tr>
<tr>
<td>Hp 2-2</td>
<td>Normal</td>
<td>9.98E+4±0.86E+4</td>
<td>8.52E-5±0.78E-5</td>
<td>0.85±0.07</td>
</tr>
<tr>
<td>Hp 2-2</td>
<td>Glycosylated</td>
<td>3.49E+4±0.48E+4</td>
<td>7.78E-5±0.60E-5</td>
<td>2.25±0.30</td>
</tr>
</tbody>
</table>

*SD of three independent preparations. \(K_d\) represents M⁻¹·sec⁻¹; \(K_d\) sec⁻¹. KD is \(K_d/K_a\).
mice with DM (33.3 ± 10.2 and 54.5 ± 26.1 nmol/L, respectively; P < 0.01 for Hp 0 and for Hp 2 for the difference with and without DM).

Tissue Iron Is Increased in Hp 0 and Hp 2 Mice and Is Further Increased by DM

Redox active chelatable iron has been demonstrated to be readily taken up by tissue culture cells.40 To determine whether the elevated catalytically redox active plasma iron found in Hp 0 and Hp 2 mice was associated with elevated deposits of tissue (kidney) iron, kidney histology was assessed. We found a profound increase in iron deposited in the proximal tubules epithelial cells of the kidney of Hp 0 and Hp 2 mice, which was significantly enhanced by diabetes (Figure 6).

Discussion

In this study, we have presented evidence in support of a novel hypothesis for why diabetic individuals with the Hp 2 allele have an increased incidence of diabetic vascular disease. We have used complementary in vitro and in vivo systems to show that the Hp 2 allele is associated with a diabetes-enhanced increase in the amount of chelatable redox active iron.

The molecular nature of this chelatable redox active iron is currently unknown. In patients with thalassemia, this iron...
component appears to be bound to albumin or citrate attributable to a saturation of the transferrin system. In the diabetic patient, this iron component may be bound to aberrantly glycated proteins or, in the case of hemoglobin, may represent iron that remains coordinated to hemoglobin but is delocalized from the hydrophobic heme pocket. Normally, heme iron sits in the hydrophobic heme pocket of hemoglobin and is neither redox active nor accessible to chelation. Oxidation of hemoglobin and conversion of Fe\(^{2+}\) (oxyHb) to Fe\(^{3+}\) (metHb) has been demonstrated to be associated with an altered orientation of Fe in the heme pocket (as assessed by x-ray diffraction) and an increase in the availability of the iron to the aqueous milieu (as assessed by an increase in the chelatability and redox activity of hemoglobin iron). A haptoglobin type–dependent role in the ability to prevent heme loss from hemoglobin has recently been demonstrated and is consistent with our data showing increased redox active iron being associated with haptoglobin 2-2-hemoglobin complexes.

The CHO cells used in these studies were not transfected with CD163. Based on our previous work using labeled Hp, the Hp-Hb complex does not enter into CHO cells. However, the Hp-Hb complex can still modulate intracellular oxidation and chelatable iron without entering the cell. The iron in Hp 2-2-Hb is not stably bound and is capable of being transferred to a variety of substrates (such as LDL) promoting oxidation\(^4\) and this process is accentuated by glycosylation of Hb. We have shown here that the increased oxidation associated with the Hp 2-2-Hb complex is inhibited by deferiprone, thereby indicating that the increased oxidative stress associated with the Hb-Hp 2-2 complex is attributable to chelatable redox active iron. In these studies, we cannot determine at what site DFO is acting as a chelator intracellularly or extracellularly. DFO may be chelating iron in the Hp 2-2-Hb complex outside of the cell and thereby preventing the release of iron from this complex that otherwise might diffuse across the cell membrane and increase intracellular oxidative stress. Alternatively, DFO may be acting intracellularly to chelate iron that has been released outside of the cell by Hp 2-2-Hb complexes and then has been taken up by the cell. This question could be answered by using high molecular weight conjugates of DFO (starch-DFO), which cannot enter cells. An alternative mechanism that must be acknowledged in explaining these studies with DFO is that DFO may be acting via a reduction mechanism independent of its chelation activity. This issue could be addressed by repeating these studies with a non–redox active iron chelator.

A key question remains the functional relevance of the increased levels of redox active iron in plasma of diabetic and nondiabetic mice with different Hp types. Histogram showing the labile chelatable plasma iron component (in nmol/L) from diabetic and nondiabetic mice genetically modified at the Hp locus. Data shown represent the mean±SEM in the different groups [n=13 in nondiabetic wild type (Hp 1), 12 in diabetic wild type (Hp 1), 5 in nondiabetic knockout (Hp 0), 5 in diabetic knockout (Hp 0), 7 in nondiabetic Hp 2, and 9 in diabetic Hp 2 mice]. B, Percentage of mice having detectable plasma labile chelatable redox active iron (LPI) in the different groups. In the absence of diabetes, 7% of the wild-type (Hp 1) mice had measurable chelatable redox active iron (LPI) and this was not significantly altered by the presence of diabetes (mean concentration 5.9±2.0 vs 8.9±3.6 nmol/L in the presence or absence of diabetes; P=NS). However, in Hp 0 40% and in Hp 2 mice 30% of the mice had measurable amounts of chelatable redox active iron (17.7±12.0 and 16.8±8.0 nmol/L, respectively). In both the Hp 0 and in the Hp 2 mice, this iron component was markedly increased with diabetes, so that 80% of the Hp 0 and 70% of the Hp 2 mice had elevated chelatable redox active iron in their plasma and the mean concentration of this iron component increased by 2- to 4-fold in the Hp 0 and Hp 2 mice with diabetes (33.0±10.2 and 54.5±26.1 nmol/L, respectively; P<0.01 for Hp 0 and for Hp 2 for the difference with and without diabetes).

### Table 2. Lipid Peroxides in CHO Cells Incubated With Hp-Hb Under High or Low Glucose Conditions

<table>
<thead>
<tr>
<th>Hp-Hb Complex</th>
<th>Low Glucose</th>
<th>High Glucose</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>2.66±0.51</td>
<td>2.67±0.47</td>
</tr>
<tr>
<td>Hp 1-1</td>
<td>2.69±0.56</td>
<td>2.64±0.47</td>
</tr>
<tr>
<td>Hp 2-2</td>
<td>2.35±0.43</td>
<td>4.98±1.15*</td>
</tr>
</tbody>
</table>

Results for lipid peroxides are expressed in nmol±SEM. All data points represent minimum of 5 separate experiments. Control represents cells incubated without haptoglobin or hemoglobin. Cell culture conditions and methods to measure the lipid peroxides are described in the online data supplement. Amount of lipid peroxide in CHO cells incubated with Hp 2-2-Hb under high glucose conditions was significantly greater than the amount of lipid peroxide obtained from cells cultured under high glucose without Hp-Hb (control) or with Hp 1-1-Hb.
Vitamin C appears to be degraded at a faster rate in Hp 2-2. Considerable interest to determine the chelatable iron component we are measuring, which is Hp- and glucose-dependent, is present even though the transferrin iron binding capacity of the serum is not saturated. It is also notable that in our in vitro cell culture paradigm, we used concentrations of Hp-Hb that were in the nmol/L range (100 ng/mL) and have shown a Hp- and glucose-dependent increase in oxidative stress in cells.

An important variable in any effect of iron is likely to be ascorbate (vitamin C). The serum level of ascorbic acid in wild-type C57Bl/6 animals has been reported to be 114 ± 3 μmol/L. We have not measured vitamin C levels in mice with different Hp types. Ascrobate concentrations have been shown to be Hp type dependent in man. The mean concentrations for Hp 1-1, 2-1, and 2-2 were reported to be 61.5 ± 16.5, 63.7 ± 10.8, and 49.9 ± 8.5 μmol/L, respectively. Vitamin C appears to be degraded at a faster rate in Hp 2-2, presumably attributable to iron mediated oxidation. Indeed, it is possible that the increased labile iron associated with Hp 2-2 may make vitamin C supplements to Hp 2-2 individuals harmful. This is supported by our recent findings in the WAVE study, in which we have shown that vitamin C results in an acceleration of atherosclerosis assessed angiographically in diabetic women with Hp 2-2.

The hypothesis we have proposed here is consistent and complementary to the hypothesis we have previously proposed as to why diabetic individuals with Hp 2-2 have greater oxidative tissue injury and increased vascular disease. We have shown in this study that hyperglycemia is associated with an increase in redox active chelatable iron within Hp 2-2-Hb complexes. It is therefore very important that Hp 2-2-Hb complexes in the diabetic state be scavenged or cleared as rapidly as possible. We have previously demonstrated that Hp 2-2-Hb complexes are cleared much more slowly than Hp 1-1-Hb complexes. Taken together these two processes result in a significantly greater potential for oxidative stress and tissue damage mediated by Hp 2-2 complexes as compared with Hp 1-1 complexes in the diabetic state.

The link between iron metabolism and cardiovascular disease in man has been elusive. As pointed out earlier, this may be attributable to the indirect manner in which iron status has been assessed. We have examined the small pool of iron which is highly labile and redox reactive. It would be of considerable interest to determine the chelatable iron component in any of the recent longitudinal studies in which we have shown a relationship between haptoglobin type and diabetic cardiovascular disease. If a direct relationship can be demonstrated between this labile iron component and diabetic vascular disease, then, by definition that this iron component is chelatable, iron chelation may emerge as a potential strategy to reduce diabetic cardiovascular disease.

**References**


