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 independent population-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{23}; 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{31} 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 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{23}; 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.

#### 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 $10^{-10}$ to $10^{-16}$ mol/L).\textsuperscript{1,39} 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{31} We found that the equilibrium dissociation constant kDa of haptoglobin for hemoglobin was approximately $10^{-16}$ 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 DCFH, or by directly measuring cellular lipid peroxides. In CHO cells cultured in DMEM media with a glucose concentration of 100 mg/dL, we found no increase in the amount of oxidative stress induced with the different Hp-Hb complexes (Hp 1-1 or Hp 2-2) as compared with cells not exposed to Hp-Hb complexes. When the CHO cells were cultured in the presence of high glucose DMEM (450 mg/dL), Hp 1-1-Hb complexes were associated with levels of oxidative stress similar to cells not exposed to Hp-Hb, however, Hp 2-2-Hb complexes induced a dramatic increase in intracellular oxidative stress assessed either by DCFH (Figure 4) or by measurement of lipid peroxides (Table 2). Although the data shown in Figure 4 and Table 2 were obtained using nonglycohemoglobin and CHO cells, similar results were obtained using either glycohemoglobin (with greater DCFH oxidation in cells incubated with glycate Hb-Hb 2-2 as compared with glycate Hb-Hb 1-1) or THP-1 cells (with greater DCFH oxidation in cells incubated with Hb-Hb 2-2 with high glucose as compared with Hb-Hb 1-1 with high glucose).

Desferrioxamine (DFO) is a high affinity iron chelator. The hyperglycemia induced change in oxidative stress with Hp 2-2-Hb complexes under high glucose conditions was completely inhibitable with DFO, suggesting that the increased oxidative stress induced by hyperglycemia in this system was attributable to an increased amount of redox active iron that is chelatable (Figure 4).

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

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±121.0 and 16.8±8.0 nmol/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 |
|-----------|------------------|------------------|------------------|
| Haptoglobin | Hemoglobin       | \(K_d \pm SD\)  | \(K_d \pm SD\) |
| Hp 1-1    | Normal           | 1.23E+5±0.17E+5 | 7.70E-5±0.39E-5 |
| Hp 1-1    | Glycosylated     | 4.27E+4±0.16E+4 | 1.06E-4±0.23E-4 |
| Hp 2-2    | Normal           | 9.98E+4±0.86E+4 | 8.52E-5±0.78E-5 |
| Hp 2-2    | Glycosylated     | 3.49E+4±0.48E+4 | 7.78E-5±0.60E-5 |

*SD of three independent preparations. \(K_d\) represents M⁻¹·sec⁻¹; \(K_d\) sec⁻¹. KD is \(K_d\)·\(\alpha\).
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. 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$^{3+}$ (methHb) 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 oxidant injury because the transferrin effectively prevents this iron from being redox active. This is why very high concentrations of transferrin are needed to act 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 diffuse across the cell membrane.

### 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>
</thead>
<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.

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 measured increased levels (60 to 70 nmol/L) of redox active iron in diabetic mice with the Hp 2 allele. Many prior studies performed in vitro investigating the toxic effects of iron have required considerably higher concentrations of iron to show a pro-oxidant effect. This is because these prior studies used free iron added to serum that contains transferrin. Until the iron binding capacity of transferrin is exceeded, the cells exposed to this iron would not be expected to show any oxidant injury because the transferrin effectively prevents this iron from being redox active. This is why very high concen-
trations of iron have been required in these systems to demonstrate pro-oxidant effects, namely to overwhelm the transferrin. The situation we have described here is quite different. 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/6J mice has been reported to be 114±3 μmol/L. We have not measured vitamin C levels in mice with different Hp types. Ascorbate 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, because by definition that this iron component is chelatable, iron chelation may emerge as a potential strategy to reduce diabetic cardiovascular disease.

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References

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Preparation of hemoglobin and glycation of hemoglobin by glycolaldehyde.

Hemoglobin was isolated from fresh human blood. Erythrocytes were washed five times with PBS and lysed with ice cold water (10 vol). Hemolysate was separated from the erythrocyte membranes by centrifugation at 15,000 g for 45 minutes. Met-hemoglobin was prepared by incubation of this hemoglobin with 1.1 mM K$_3$Fe(CN)$_6$ per 1 mM hemoglobin for 30 minutes at room temperature. The met hemoglobin was then purified using PD-10 columns. Oxy and met hemoglobin were glycated in vitro using glycolaldehyde.$^1$ 10 mM hemoglobin was incubated with 25 mM glycolaldehyde, 1.5 mM phenylmethylsulfonyl fluoride (PMSF), 1% pencillin-streptozocin and 40 ug/ml gentamicin for three days at 37°C. Hemoglobin was then dialyzed in PBS for 48 hours. In all cases when comparing glycated to non-glycated hemoglobin in this study we have used hemoglobin that was treated identically in all respects for both groups except for the inclusion of glycolaldehyde (i.e., both the glycated and non glycated hemoglobin were incubated at 37°C for three days and then dialyzed). For each experiment we prepared fresh glycated and non-glycated hemoglobin using the same method.

Measurement of the affinity of hemoglobin for haptoglobin.

The affinity of normal and glycated hemoglobin for haptoglobin 1-1 and haptoglobin 2-2 was measured by surface-plasmon resonance (SPR) analysis using the BIAcore 3000 instrument (BIAcore, Upsalla, Sweden).$^2$ We prepared human haptoglobin 1-1 or human haptoglobin 2-2 coupled to the BIAcore sensor CM5 chip as follows. The BIAcore sensor CM5 chip was activated with a 1:1 mixture of 0.2 M N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxysuccinimide at a flow rate of 10 ul/min for seven minutes according to the manufacturer’s instructions. Immobilization of the haptoglobin 1-1 or 2-2 on two different activated chips was then achieved by injecting haptoglobin at a concentration of 30 ug/ml in 10 mM sodium acetate (pH 4.5), and the remaining binding sites on the chip were blocked with 1M ethanolamine (pH 8.5). The SPR signal from immobilized haptoglobin generated 3-5x10$^3$ BIAcore response units (RU) which is equivalent to 20-30 fmol/mm$^2$. We demonstrated that the measured association was limited by the rate of actual binding of haptoglobin to hemoglobin rather than being limited by flow using the mass transfer limitation test by showing that increasing the flow rate did not
increase the measured binding. The binding affinity was studied by injecting different concentrations (25-200 nM) of normal or glycosylated Hb in 10 mM phosphate buffered saline, pH 7.4 at 37°C. Each injection was followed by a regeneration step with 10 mM glycine-HCl, pH 2.5. Data analysis and calculation of kinetic constants was performed using BIAevaluation software (3.1). A simple 1:1 langmuir association/dissociation model was employed to fit the data.³

**Measurement of redox active chelatable iron.**

We have used the method of Cabantchik et al to measure the amount of redox active chelatable iron, also known as labile plasma iron or LPI, in a given solution or in plasma.⁴ Briefly, dihydrorhodamine (DHR) was used as a sensitive fluorescent indicator of oxidative activity. In the assay to measure redox active chelatable iron each solution or serum sample was tested under two different conditions: with 40 uM ascorbate alone and with 40 uM ascorbate in the presence of 50 uM 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. For the assay, quadruplicates of 20 ul of test solution were transferred to clear bottom 96 well plates. To two of the wells 180 ul of iron free Hepes-buffered saline containing 40 uM of ascorbate and 50 uM of the DHR was added. To the other two wells, 180 ul of the same solution containing the iron chelator (50 uM) was added. Immediately following the addition of reagent, the kinetics of fluorescence increase were followed at 37°C in a BMG GalaxyFlouroStar microplate reader with a 485/538 nm excitation/emission filter pair, for 40 minutes, with readings every 2 minutes. The slopes of the DHR fluorescence intensity with time were then determined from measurements taken between 15-40 minutes. The free redox active iron concentration (in uM) was then determined from calibration curves relating the difference in slopes with and without chelator vs. Fe concentration. Calibration curves were obtained by spiking plasma-like media or hepes-buffered saline with Fe:nitrilotriacetic acid (NTA) to give a final concentration of 40-100 uM followed by serial dilution.

**Measurement of lipid peroxides.**

Cellular lipid peroxides were measured in CHO cells incubated with Hp-Hb complexes (100ng/ml) under conditions of low (100mg/dl) or high (450mg/dl) glucose DMEM for 16 hours.⁵ CHO cells (80% confluent) in a 6 well tissue culture
dish (corning) were washed once with PBS. The cells were then extracted three times (2 cc for 15 minutes for each extraction) with isopropanol:hexane (2:3). This extract was collected in a glass tube. 1 cc of acidified dH2O (200 H2O:1 conc H2SO4) was then added to this extract in the glass tube and the mixture vortexed. The upper phase was then transferred to a new glass tube and the liquid left to evaporate overnight. To the dry tube, 1 cc of lipid peroxide reagent was then added (0.2 M potassium hydrophosphate, 0.12 M potassium iodide, 0.15 M sodium azide, 2g/l igepal, 0.1g/l alkylbenzyldimethyl ammonium chloride, 10 uM ammonium molybdate). After a 30 minute incubation in the dark the OD of the sample was measured at 365 nM. The amount of nmol of lipid peroxides in a given sample under the reaction conditions defined above was derived using the known extinction coefficient for lipid peroxides (2.46x10^4 M^-1) using the relationship nmol lipid peroxides=measured (OD_{365})x(100)/2.46.

References for on-line supplement:


