Genetically Determined Heterogeneity in Hemoglobin Scavenging and Susceptibility to Diabetic Cardiovascular Disease

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Abstract—A major function of haptoglobin (Hp) is to bind hemoglobin (Hb) to form a stable Hp-Hb complex and thereby prevent Hb-induced oxidative tissue damage. Clearance of the Hp-Hb complex can be mediated by the monocyte/macrophage scavenger receptor CD163. We recently demonstrated that diabetic individuals homozygous for the Hp 2 allele (Hp 2-2) were at 500% greater risk of cardiovascular disease (CVD) compared with diabetic individuals homozygous for the Hp 1 allele (Hp 1-1). No differences in risk by Hp type were seen in individuals without diabetes. To understand the relationship between the Hp polymorphism and diabetic CVD, we sought to identify differences in antioxidant and scavenging functions between the Hp types and to determine how these functions were modified in diabetes. The scavenging function of Hp was assessed using rhodamine-tagged and 125I-Hp in cell lines stably transfected with CD163 and in macrophages expressing endogenous CD163. We found that the rate of clearance of Hp 1-1–Hb by CD163 is markedly greater than that of Hp 2-2–Hb. Diabetes is associated with an increase in the nonenzymatic glycosylation of serum proteins, including Hb. The antioxidant function of Hp was assessed with glycosylated and nonglycosylated Hb. We identified a severe impairment in the ability of Hp to prevent oxidation mediated by glycosylated Hb. We propose that the specific interaction between diabetes, CVD, and Hp genotype is the result of the heightened urgency of rapidly clearing glycosylated Hb-Hp complexes from the subendothelial space before they can oxidatively modify low-density lipoprotein to atherogenic oxidized low-density lipoprotein. (Circ Res. 2003;92:1193-1200.)

Key Words: cardiovascular disease ■ diabetes ■ hemoglobin ■ haptoglobin ■ scavenger receptor

There exists a growing body of evidence that diabetic vascular disease develops only in those patients who are genetically susceptible.¹ There exists two classes of alleles at the haptoglobin (Hp) locus, denoted class 1 and class 2, and three potential genotypes denoted Hp 1-1, Hp 2-1, and Hp 2-2.² We have recently demonstrated in several independent longitudinal prospective studies that this polymorphism in the Hp gene is an independent risk factor for cardiovascular disease (CVD) in the diabetic individual.³ Specifically, individuals who were found to be homozygous for the Hp 2 allele (Hp 2-2) were found to be at significantly greater risk of developing myocardial infarction, stroke, and cardiovascular death. No relationship in these studies was found between the Hp polymorphism and the risk of CVD in the nondiabetic individual. The mechanism whereby the Hp polymorphism confers susceptibility to CVD and the reason this interaction is specific to the diabetic state is not understood.

Hp circulates in plasma as a polymer whose stoichiometry and biophysical properties are Hp genotype dependent (hereafter referred to as Hp 1-1, Hp 2-1, or Hp 2-2 protein).² All three types of Hp protein (Hp 1-1, Hp 2-1, and Hp 2-2) bind free hemoglobin (Hb) equally well with high affinity, thereby serving to prevent iron loss and protecting tissues from Hb-induced oxidative damage.² Hp also seems to have immunomodulatory functions intimately related to its Hb binding function, because the Hp-Hb complex binds with high affinity to the CD163 scavenger receptor present on monocytes and macrophages.⁴ Several differences have been identified between Hp 1-1, Hp 2-1, and Hp 2-2 with regard to these antioxidant and CD163 binding functions. First, we have demonstrated that Hp 1-1 protects low-density lipoprotein (LDL) against Hb-induced oxidation better than Hp 2-2.⁵ Second, Kristiansen et al⁶ have demonstrated that Hp 2-2 binds with a 10-fold higher affinity to CD163 compared with Hp 1-1. However, these functional differences between the different Hp types do not seem to be specific for the diabetic state.

In this study, we have sought to additionally understand functional differences between the different Hp types with regard to their antioxidant and immunomodulatory function and to understand why these differences may only be manifested clinically in the diabetic state. Diabetes is associated with a marked increase in the nonenzymatic glycosylation of numerous proteins, including Hb. We demonstrate here that
the oxidative activity of glycosylated Hb is not effectively blocked by Hp and that there is a difference in the rate of clearance of the different types of Hp-Hb complexes by CD163. We propose that the interaction between these processes results in Hp type differences in the amount of oxidative stress and consequently oxidative modification of proteins such as LDL within the blood vessel wall of the diabetic patient.

Materials and Methods

Reagents
CHO-CD163 cells were generated by Soren Moestrup and represent CHO cells stably transfected with human CD163 constitutively expressed from a CMV promoter. FITC-CD14, RPE-streptavidin, and CD163 mAb were purchased from Serotec. Biotinylated CD163 Ab was prepared by Enco diagnostics. Hp was purified from healthy volunteers using a rabbit polyclonal antibody affinity column and was more than 99% pure based on gel electrophoresis and Coomassie blue staining. Radiochemicals were from Amersham. LDL was more than 99% pure based on gel electrophoresis and Coomassie blue staining. Radiochemicals were from Amersham. LDL was prepared from human plasma as previously described. Hb was prepared from human red blood cells as previously described. All other chemicals were purchased from Sigma.

Labeling of Haptoglobin
Hp purified from human plasma using a polyclonal rabbit antibody affinity column was labeled with both 125I and with rhodamine. Hp was radio-iodinated using the chloramines-T method. Hp 5 µg (Hp 1-1 or Hp 2-2) was mixed with 50 µL NaH2PO4, 0.5 mCi 125I, and 5 µL chloramine-T (2 mg/mL) and stirred for 45 seconds at room temperature. The reaction was terminated with 12.5 µL Na2S2O5 (2 mg/mL). Subsequently, the labeled protein was purified using a PD-10 column (Amersham) with PBS containing 1% BSA as the running buffer. The typical specific activity was 70 000 to 90 000 cpm/ng protein. Hp was labeled with rhodamine (Molecular Probes) according to the manufacturer’s protocol.

Cell Culture
CHO cells were grown in F12 medium with 10% FBS. For binding assays, the medium was changed to 0.5% FBS. For IP3 assays, the medium was changed to F12 medium without inositol overnight. Undifferentiated THP-1 cells were grown in suspension with RPMI with 10% FBS. The THP-1 cells were differentiated by treatment with PMA (10 ng/mL) for 4 days and 2.5×10−7 mol/L dexamethasone for 1 day.

Western Blot for CD163
CD163 protein expression was assessed by Western blot on total cell extracts from CHO-CD163 cells and THP-1 cells using a monoclonal Ab to human CD163 (Serotec). Cell extracts were prepared using NP-40 lysis buffer (0.5% NP-40). A rabbit anti-mouse IgG alkaline phosphatase antibody was used as the secondary antibody.

Binding Assay
In CHO cells, the assay was performed in 24-well plates. In a typical experiment, 4.5×104 cpm was added to the CD163-CHO cells (80% confluent) for 16 hours at 4°C in 200 µL of 140 mM NaCl, 10 mM HEPES, 5 mM L-CaCl2, 0.5 mM L-MgCl2, and 1% BSA. A variable amount of unlabeled competitor Hb-Hb complex was added to the cells 60 minutes before the addition of the labeled Hb-Hb complex. Hb (concentration reported for the dimer that is the form of Hb found at the concentrations used in this study) was used in all experiments in a 2-fold molar ratio relative to the concentration of Hp monomer to assure that all Hb was fully saturated with Hb. After the 16-hour incubation period, the cells were washed three times with PBS with 1% BSA and bound radioactivity was released on the addition of 500 µL of 1% SDS and quantitated by γ scintillation counting. Nonspecific binding was determined from the amount of binding of the radioactive Hp-Hb complex to CHO-CD163 cells in the presence of a 1000-fold excess of cold Hb-Hb complex. Nonspecific binding (5% to 10% of the total binding) was subtracted from the total binding to obtain the specific binding. The Kd of binding for each Hp type was determined as the concentration of Hp polymer producing half-maximal specific binding. This concentration was based on the known polymeric composition and average molecular mass of Hp 1-1 and Hp 2-2 polymeric molecules.

Endocytosis Experiments
In all experiments involving labeled Hp protein, Hp 1-1 or Hp 2-2 was studied at the same time in parallel cultures and was used at the same specific activity.

Uptake of Fluorescently Labeled Hp-Hb Complex
Hp (1-1) and Hp (2-2) were fluorescently labeled with rhodamine Red-X dye, and the concentration and coupling efficiency of both were determined and adjusted to be equivalent when used in experimental studies. CD163-transfected and control CHO cells were seeded into 4-well Laboratory-Tek chamber slides (2×104 cells/well) and incubated for 36 hours at 37°C. After washing twice with PBS (1% BSA), the cells were incubated with 4µCi NaClO2 incubator for up to 5 hours. As discussed above for the binding studies, the stoichiometric ratio of Hp to Hb was kept at 1:2 to ensure that all Hp was saturated with Hb. Cell-associated radioactivity was assessed after washing the cells with PBS-1% BSA by lysing the cells with 1 mL of 1% SDS and subjecting the solubilized cell extract to γ scintillation counting. Surface receptor binding was measured and subtracted from the experimental values (total cell-associated counts per minute) by repeating the previous experiments at 4°C rather than 37°C, thereby providing a measurement of the amount of counts per minute that was internalized.

The techniques described above to study endocytosis reveal only the steady-state level of the Hp-Hb complex, which represents the difference between the rate of uptake of the complex (endocytosis) and the rate of degradation of the complex. To examine the rate of uptake, we blocked degradation of the complex with the lysosomal inhibitor chloroquine (50 µmol/L). The total amount of cell-associated radioactivity was assessed at time intervals after the addition of chloroquine to calculate a rate of uptake. Degradation rate was assessed by first incubating the radiolabeled complex with the cells for 1.5 hours and then changing the solution bathing the cells so that no additional uptake of radiolabeled complex could occur. This point was taken as the zero time point, and the decrease in cell-associated counts per minute was measured as a function of time.

Activation of the CD163 Receptor by Hp-Hb

Ip3 Assay
CD163-transfected CHO cells were initially grown in 6-well plates in F12 with 10% FBS. The cells were then cultured for 16 hours in inositol-free F-12 medium (Beta-Emek) supplemented with 0.5% FBS, 2 mM glucose, and 1 µCi/mL (myo-1H) inositol (Amersham). The cells were then washed twice with F-12 medium containing 50 mM inositol, 20 mM HEPES and 20 mM LiCl and preincubated with this same solution for 10 minutes at 37°C. The production of IP3 in these cells was assessed 60 minutes after Hp-Hb complex,
or Hp without Hb (100 nmol/L final concentration in all cases) was added to the cells. The reaction was terminated by extracting the cells with 2 mL ice-cold chloroform/methanol (1:2 vol/vol). Equal volumes of water and chloroform were then added to the chloroform/methanol solution. The tubes were then vortexed and centrifuged at 800 g for 10 minutes. From the upper aqueous phase, IP₃ was extracted using ion-exchange chromatography on an AG1-8 formate resin column (Bio-Rad).⁷

**Measurement of Free Intracellular Calcium Mobilization**

CD163-transfected CHO or THP-1 cells were grown on glass coverslips and loaded with Fura-2. See the online data supplement for a detailed description of cell washing and incubation conditions. Raw data were recorded as the ratio of fluorescence emitted at 510 nm after sequential excitation at 340 and 380 nm (R₅₅₀=F₃₄₀/F₃₈₀, where F₃₄₀ and F₃₈₀ indicate fluorescence counts after excitation at 340 and 380 nm, respectively).⁸

**Preparation of Glycosylated Hemoglobin and Oxidation of LDL**

Met-Hb was prepared as previously described.² Hb was glycosylated in vitro using glycolaldehyde (Fluka AG) as previously described.⁹–¹¹ The oxidation of LDL was performed and assessed as previously described³ using a fixed concentration of Hb and up to 2-fold excess of Hp.

**FACS Analysis of CD163 Expression on Monocytes of Patients With and Without Diabetes**

FACS analysis was performed on 100 μL of heparinized blood obtained from 53 ambulatory diabetic and 53 ambulatory nondiabetic (by history) volunteers. Red cells were lysed using NH₄Cl red cell lysis solution (Sigma). Monocytes were identified by FACS using a CD14-FITC–conjugated antibody, and CD-163–positive cells were identified using CD163-biotinylated antibody and RPE-streptavidin–conjugated antibody. All antibodies were used at a 1:500 dilution, whereas the RPE-streptavidin was used at a 1:1000 dilution as obtained from the manufacturer. Fixation was performed with 1% (final) paraformaldehyde. Results were expressed as the percentage of CD14⁺ cells, which also express CD163.

**Statistics**

Differences between groups were compared by the students t test. Results are reported as mean±SEM.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

**Results**

**Specific Binding of Hp-Hb to CD163**

We sought to demonstrate specific binding of the Hp-Hb complex to CD163 using CHO cells constitutively expressing CD163 at high levels (Figure 1A). We found no specific binding associated with Hb or Hp alone to CHO-CD163 cells and no specific binding of the Hp-Hb complex to CHO cells, as the difference between the amount of binding of these ¹²⁵I-labeled ligands with and without a 1000-fold excess of cold ligand (specific binding). This experiment was repeated 10 times, with values shown reflecting the mean±SEM. The difference in the specific binding between Hp 1-1–Hb and Hp 2-2–Hb complexes was statistically significant (P<0.0001), C. Determination of the K₅ for CD163 for Hp 1-1–Hb and Hp 2-2–Hb complexes. The K₅ was calculated as the concentration of cold competitor (expressed as the concentration of Hp polymer) resulting in a 50% reduction in specific binding. The mean K₅ of Hp 1-1–Hb was determined to be 1.9 nmol/L, and the mean K₅ of the Hp 2-2–Hb complex was determined to be 0.25 nmol/L (based on 8 independent experiments, P<0.0001 for the K₅ between Hp 1-1 and Hp 2-2).
Figure 2. Endocytosis. A, Rhodamine. Hp 1-1 or Hp 2-2 was labeled with rhodamine, and the uptake (with and without complex formation with Hb) into CHO and CHO-CD163 cells was followed by fluorescent microscopy. B, Cell-associated $^{125}$I-Hp. Steady-state level (counts per minute) of cell-associated $^{125}$I-Hp 1-1–Hb and $^{125}$I-Hp 2-2–Hb in CHO-CD163 cells at 37°C. Total counts per minute represent both cell-surface binding and internalization (endocytosis). The internalized count per minute values were calculated as the difference between the values obtained in parallel studies done at 4°C (reflecting binding only) and 37°C (reflecting binding and internalization). Values shown represent mean±SEM of 6 independent experiments. There was a statistically significant difference in the internalization of Hp 1-1–Hb from Hp 2-2–Hb at all time points shown beginning with the 40-minute time point ($P<0.02$ for each time point). C, Measurement of rate of uptake of Hp-Hb. Lysosomal degradation of Hp-Hb was inhibited using chloroquine. The amount of internalized Hp-Hb complex at each time point was calculated by taking the difference between the values obtained at 4°C and 37°C, as described for panel B. This experiment was repeated 6 times. There was a statistically significant difference evident in the uptake of Hp 1-1–Hb versus Hp 2-2–Hb for all time points shown, beginning with the first time point tested at 15 minutes ($P<0.02$). D, Measurement of degradation rate of the Hp-Hb complex. The Hp-Hb complex was incubated with CHO-CD163 cells for 1.5 hours to permit uptake of the complex, and then the medium was changed. The time-dependent decay of the complexes was then compared, taking the zero time point as 100% value after the initial loading. There was no difference in the time to reach 50% of the initial value between Hp 1-1 and Hp 2-2 (50 minutes). The experiment was repeated four times, and values shown represent the mean±SEM for each time point. E, Measurement of internalized $^{125}$I-Hp in THP-1 macrophages. As described above, internalized values were calculated as the difference between that obtained at 37°C and 4°C. Uptake of Hp-Hb was significantly greater in THP-1 cells treated with dexamethasone compared with THP-1 cells not treated with dexamethasone. Uptake of Hp 1-1–Hb was significantly enhanced compared with Hp 2-2–Hb (1.5-fold at 2 hours, $P<0.0001$ based on three independent studies).
which had not been transfected with CD163 (Figure 1B). The CD163 Hp-Hb complex interaction was found to be of extremely high affinity with a $K_d$ in the nanomolar range. Consistent with prior studies, we found that the affinity of CD163 for the Hp-Hb complex in these cells was ≈8-fold higher for Hp 2-2 compared with Hp 1-1 (Figure 1C).

Receptor-Mediated Endocytosis of the Hp-Hb Complex by CD163

We sought to demonstrate specific uptake of the Hp-Hb complex in CHO-CD163 using two different approaches. First, we coupled rhodamine to Hp and followed uptake using fluorescence microscopy. No uptake of the rhodamine-tagged Hp-Hb complex was evident in untransfected CHO cells (Figure 2A), nor was uptake evident using rhodamine-tagged Hp alone in the CD163-CHO cells (Figure 2A). Unexpectedly, we observed a time- and temperature-dependent qualitative increase in uptake of rhodamine-tagged Hp 1-1–Hb complex compared with rhodamine-tagged Hp 2-2–Hb complex (Figure 2A). Second, we used a more quantitative approach to assess endocytosis using I$^{125}$-labeled Hp with or without Hb. We found, similar to our binding studies, that there was no uptake of Hp alone or in CHO cells lacking CD163. In CHO-CD163 cells we found similar to our studies with rhodamine-tagged Hp that there was significantly more internalization of the Hp 1-1–Hb complex compared with the Hp 2-2–Hb complex (Figure 2B).

The steady-state level of complex (counts per minute), as shown in Figure 2B, is a reflection of both the rate of uptake of the complex and the rate of intracellular lysosomal degradation of the complex. We inhibited degradation of the complex using chloroquine and thereby were able to examine the rate of uptake of the complex. We found that the rate of uptake of the Hp 1-1–Hb complex was significantly greater than that of the Hp 2-2–Hb complex (Figure 2C). To study the rate of degradation of the complex, we incubated the CHO-CD163 cells with $^{125}$I-Hp–Hb complex for 1.5 hours to allow the complex to be internalized and washed the cells extensively to prevent any additional uptake of labeled complex. The decay of the complex (counts per minute) was then followed as a function of time to determine the half-life of the complex. We found that the rates of degradation of the Hp 1-1–Hb and Hp 2-2–Hb complexes were not different from one another (Figure 2D). Taken together, these studies suggest that the higher steady-state levels of internalized Hp 1-1–Hb complex compared with the Hp 2-2–Hb complex are attributable to an increased rate of uptake of Hp 1-1–Hb.

The expression of CD163 is normally restricted to monocyte/macrophage cells. We sought to show that the uptake of the Hp-Hb complex and the apparent preference for Hp 1-1 was manifested in macrophages as well. No cell lines have been previously described that produce CD163 except SU-DHL-1, which is reportedly of T cell lineage. CD163 is known to be increased in macrophages relative to monocytes and is additionally induced by dexamethasone. We therefore differentiated the human promonocytic leukemia cell line THP-1 with PMA and then with dexamethasone and found by Western blot a dramatic increase in the expression of CD163 (Figure 1A). We did not find a significant uptake of Hp or the Hp-Hb complex into undifferentiated THP-1 cells. However, similar to that described above for the CHO-CD163 cells, we observed markedly more uptake of the Hp 1-1–Hb complex compared with the Hp 2-2–Hb complex (Figure 2E).

Activation of CD163 by the Hp-Hb Complex

Previous studies using a monoclonal antibody to CD163 demonstrated that cross-linking of CD163 led to an increase in Ca$^{2+}$ mobilization, IP3 production, and inflammatory cytokine production (interleukin-1, interleukin-6, and granulocyte-macrophage colony stimulating factor). It was proposed but never demonstrated that the polymeric Hp-Hb complex might also effectively cross-link the receptor and lead to activation of these processes. We set out to determine if indeed the Hp-Hb complex could activate these...
Hb (Figure 3B). The time to activation was shorter and the frequency of activation was greater when using Hp 2-2 events was varied from 2.5 to 20 μmol/L. Data shown represent the mean ± SEM of 4 independent experiments. Data are expressed as a percentage inhibition of the increase in Hb-induced LDL oxidation obtained in the absence of Hp.

Processes via CD163 and if there were differences between the different Hp types.

IP3 production in the CHO-CD163 cells was assessed after stimulation of H1-inositol–loaded cells with Hp-Hb. We found a rapid and transient increase in IP3 production after the addition of Hp-Hb complex that was markedly greater for Hp 2-2 complexes compared with Hp 1-1 complexes. We found no increase in IP3 with Hp alone (Figure 3A).

We examined Ca2+ mobilization in CHO-CD163 cells exposed to Hp-Hb using methodology we have previously described in detail.8 We found no mobilization by Hp alone, nor did we find mobilization in CHO cells lacking CD163 (Figure 3B). However, we observed a specific rapid and transient flux of Ca2+ in CHO-CD163 cells in response to the Hp-Hb complex (Figure 3B). Moreover, we found that the lag time to activation was shorter and the frequency of activation events was greater when using Hp 2-2–Hb compared with Hp 1-1–Hb (Figure 3B).

Antioxidant Activity of Haptoglobin Against Glycosylated Hemoglobin

We have previously described in detail an assay to measure the ability of Hp to inhibit Hb-induced peroxidation of LDL and linoleic acid.5 Diabetes is associated with a marked increase in the nonenzymatic glycosylation of numerous proteins, including Hb. We glycosylated Hb in vitro using glycoaldehyde and found a marked impairment in the ability of Hp to block the Hb-induced oxidation of LDL by glycosylated Hb. Consistent with our previous findings, stoichiometric amounts of Hp (1:1 molar ratio of Hp monomer to Hb dimer) were found to inhibit >90% of the oxidation of LDL by Hb that had not been glycosylated.5 However, Hp (even when present in molar excess) was capable of inhibiting only 10% of the oxidation induced by glycosylated Hb (Figure 4).

Figure 4. Oxidation of LDL by Hb. Hb was glycosylated and used to oxidize LDL, as described in Materials and Methods. The concentration of Hb (glycosylated or nonglycosylated) used in these studies was 10 μmol/L. The concentration of Hp 1-1 or Hp 2-2 was varied from 2.5 to 20 μmol/L. Data shown represent the mean ± SEM of 4 independent experiments. Data are expressed as a percentage inhibition of the increase in Hb-induced LDL oxidation obtained in the absence of Hp.

Figure 5. Fluorescence-activated cell sorting for CD163. Histogram showing the mean percentage of CD14-positive cells, which also were positive for CD163 in individuals with or without diabetes (n=53 in both groups). The mean in nondiabetic individuals was 7.2±0.9%, and the mean in diabetic individuals was 3.5±0.6% (P<0.0007).

Expression of CD163 on Monocytes From Diabetic Patients

Reports from several laboratories have suggested that ≈5% of all circulating monocytes express CD163 as detected by FACS.15,17 We sought to determine if this might be altered in the diabetic state. We performed FACS for CD14 and CD163 using fresh blood taken from healthy ambulatory individuals with and without diabetes to determine the percentage of monocytes expressing CD163. There was no difference in the demographic (age and sex) and relevant clinical characteristics of the two groups (ie, active smoking) except for the presence or absence of diabetes. We found a profound and consistent statistically significant reduction in the percentage of monocytes expressing CD163 in patients with diabetes as assessed by FACS (Figure 5).

Discussion

The oxidative modification hypothesis of atherosclerosis states that oxidative modification of LDL or other oxidative events within the blood vessel wall promotes the development and progression of atherosclerotic lesions.18,19 Hb is a potent potential oxidant of LDL by the Fenton reaction.21 Hb is released from red cells with intravascular hemolysis and at sites of endothelial cell injury may enter into the subendothelial space.22 The amount of Hb penetrating into the blood vessel wall is increased in diabetes because of an increased turnover of red cells23 and an increase in endothelial cell dysfunction and injury.24 Hp is normally found in serum in a >400 molar excess compared with free Hb,25 and therefore all intravascular free Hb will be rapidly bound by Hp, preventing Hb-induced oxidation. However, we have shown that the antioxidant function of Hp is impaired against the glycosylated fraction of Hb (GlyHb), a fraction that is markedly increased in the diabetic state. Even though the GlyHb can still bind to Hp to form a GlyHb-Hp complex, the oxidative activity of GlyHb is not completely blocked by the binding of Hp. Therefore, the GlyHb-Hp complex can continue to oxidatively modify proteins such as LDL within the vessel wall. It is therefore of heightened importance in the diabetic state, where the amount of GlyHb-Hb complexes in
the vessel wall is increased because of both an increase in GlyHb and an increase in endothelial injury, to capture and clear Hp-Hb complexes from the vessel wall as rapidly as possible to minimize oxidative damage. We have demonstrated here that Hp 1–1:Hb complexes are more rapidly cleared than Hp 2–2:Hb complexes, which we propose would result in significantly less oxidative damage within the vessel wall in diabetic individuals with Hp 1–1 compared with diabetic individuals with Hp 2–2.

The ability of Hp to prevent Hb-induced oxidation has been shown to be the result of increased stabilization of the heme group within the Hb. Glycosylated Hb seems to release free iron more readily than nonglycosylated Hb. Why does glycosylation of Hb interfere with the ability of Hp to inhibit the oxidative activity of Hb? Nonenzymatic glyco-
sylation can occur on any of the N-terminal α NH2 groups on the α or β chain of Hb or on any of the lysine ε amino groups from these chains, and this process is markedly increased in the diabetic state. Two of the six lysine residues in Hb that are preferentially glycosylated have been directly implicated in binding to either the heme group (where the lysine ε amino group of lys66 of the β chain of Hb is believed to make contact with a propionic acid carbonyl of the heme) or to Hp (where lys17 of the β chain of Hb has been shown to be critical for the binding of Hb to Hp).

We have identified several additional mechanisms that may be important in understanding the mechanism whereby the protein products of the two different Hp alleles confer differential susceptibility to diabetic CVD. First, the Hp types differ dramatically in their shape and size and consequently in their sieving potential to enter the subendothelial space from the serum and bind free Hb that may be released at sites of vascular injury directly into the vessel wall. Second, the ability to capture the Hp-Hb complex is dependent on the number of cells expressing CD163 and the receptor density. We have demonstrated that there is a marked decrease in the percentage of monocytes expressing membrane-associated CD163 in individuals with diabetes. Third, the activated macrophage is a well-recognized source of cytokines and proteases that contributes to the atherosclerotic process. We have demonstrated marked differences between the two Hp types in two distinct markers of macrophage activation, which are known to result in the increased production of inflammatory cytokines implicated in the atherosclerotic process.

The model we have developed here involving increased Hb-induced oxidative stress in the vessel wall in diabetic individuals with Hp 2–2 would suggest that antioxidant therapy may be efficacious in diabetic individuals with Hp 2–2. We have recently found in diabetic Hp 2–2 participants in the HOPE study that vitamin E did not reduce the primary composite outcome cardiovascular outcome overall but may have reduced two components of this outcome, myocardial infarction and death from CVD (A.P. Levy et al, unpublished data, 2003). The model we have presented here is consistent with these findings and provides a simple in vitro and in vivo paradigm in which to test therapeutic interventions to reduce diabetic CVD attributable to the Hp polymorphism.

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On-line data supplement for method of measurement of free intracellular calcium mobilization.

CD163 transfected CHO or THP-1 cells were grown on glass coverslips and loaded with 5 μM Fura-2 acetoxymethyl ester. (Molecular probes) for 25 minutes at room temperature in physiological Tyrode’s solution (126 mM NaCl, 2 mM CaCl₂, 4.4 mM KCl, 1 mM MgCl₂, 18 mM NaHCO₃, 11 mM glucose, 4 mM HEPES) containing 2% BSA. Cells were washed twice with Tyrode’s solution to remove excess Fura-2 and then transferred to a non-fluorescent chamber mounted on the stage of an inverted microscope (Diaphot 300, Nikon). The chamber was perfused with Tyrode’s solution (containing Hp alone or Hp-Hb complex) at a rate of 1 ml/min and experiments were performed at 37°C. Fluorescence recordings were measured using a dual-wavelength system (Delta-Scan Photon Technology Intl.).