**PRECLINICAL STUDY**

# Haptoglobin Genotype Determines Myocardial Infarct Size in Diabetic Mice

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| **Objectives** | We sought to understand the importance of oxidative stress in explaining why the haptoglobin (Hp) genotype determines myocardial infarction (MI) size in diabetes mellitus (DM). |
| **Background** | Two common alleles (1 and 2) exist at the Hp locus in humans. The Hp 2 allele is associated with increased MI size in individuals with DM. In vitro, the Hp 2 protein is associated with increased generation of oxidatively active iron, whereas the Hp 1 protein is associated with increased production of the antioxidant cytokine interleukin (IL)-10. |
| **Methods** | Myocardial infarction was produced by myocardial ischemia-reperfusion (IR) in DM C57BL/6 mice carrying the Hp 1 or Hp 2 allele. Myocardial oxidative stress after IR was assessed using electrospray ionization mass spectrometry. Redox active iron and IL-10 were measured in the serum after IR. |
| **Results** | Myocardial infarction size was significantly larger in Hp 2 mice as compared with Hp 1 mice (44.3 ± 9.3% vs. 21.0 ± 4.0%, p = 0.03), and these larger infarctions were associated with a significant increase in a panel of hydroxyl-eicosatetraenoic acids. Redox active iron was greater in Hp 2 mice (0.45 ± 0.11 $\mu$mol/l vs. 0.14 ± 0.05 $\mu$mol/l, p = 0.02), whereas IL-10 was greater in Hp 1 mice (85.8 ± 12.9 pg/$\mu$l vs. 46.7 ± 10.8 pg/$\mu$l, p = 0.04) after IR. Administration of an antioxidant (BXT-51072) to Hp 2 mice reduced myocardial injury after IR by more than 80% (p = 0.003), but no myocardial protection was provided by the antioxidant to Hp 1 mice. |
| **Conclusions** | The increased MI size in DM Hp 2 mice occurring after IR may be due to increased oxidative stress.    (J Am Coll Cardiol 2007;49:82–7) © 2007 by the American College of Cardiology Foundation |

Ischemia-reperfusion (IR) plays an important role in determining the amount of myocardial injury occurring in acute coronary syndromes (1). Ischemia-reperfusion injury is increased in diabetes mellitus (DM) because of increased oxidative stress (2) and an exaggerated inflammatory reaction (3). Functional polymorphisms in genes that modulate oxidative stress and the inflammatory response may therefore be of heightened importance in determining infarct size in DM.

The haptoglobin (Hp) gene locus at chromosome 16q22 is polymorphic with 2 common classes of alleles denoted 1 and 2 (4). We have reported that DM individuals with the Hp 2 allele have significantly larger myocardial infarctions (MIs) than DM individuals homozygous for the 1 allele (5). We have proposed that this may be due to differences in the antioxidant and anti-inflammatory properties of the Hp 1 and Hp 2 proteins (6–11). To test this hypothesis, we assessed a variety of oxidative and inflammatory parameters occurring after IR in DM mice genetically modified at the Hp locus. Moreover, we have directly tested the importance of oxidative stress in mediating myocardial injury in these mice with oral administration of an antioxidant.

## Materials and Methods

**Animals.** The protocols used in these studies were approved by the Technion Faculty of Medicine Animal Care

and Use Committee. Wild-type C57BL/6 mice carry only a class 1 Hp allele highly homologous to the human Hp 1 allele and are referred to as Hp 1 mice (12). The Hp 2 allele exists only in humans (4). Mice containing the Hp 2 allele were generated by introducing the human Hp 2 allele as a transgene in a C57BL/6 Hp knockout genetic background (13–15).

**Diabetes.** Diabetes was induced by an intraperitoneal injection of 200 mg/kg streptozotocin in 3-month-old mice. The severity of diabetes was defined both by spot non-fasting glucose (glucometer) and hemoglobin (Hb)A1c (Helena Diagnostics, Netanya, Israel). Myocardial infarction was produced approximately 1 month after injection of streptozotocin.

**Myocardial IR model.** We used a modification of a previously described IR model (16). Mice were anesthetized with a mixture of ketamine (150 mg/kg) and xylazine (9 mg/kg) and body temperature was maintained at 37°C using a heating pad. The trachea was intubated with a 21-G needle that was previously decapitated and had a blunt end. The tube was connected to a respirator (Model 687, Harvard Apparatus, Halliston, Massachusetts). The respirator tidal volume was 1.2 ml/min and the rate was 100 strokes/min. A left lateral thoracotomy was made in the fourth intercostal space; the skin, muscles, and ribs were retracted; and the pericardial sac was removed. Ligation of the left anterior descending coronary artery (LAD) was made using a 7/0 Ethicon virgin silk, non-absorbable suture, connected to a micropoint reverse-cutting 8-mm needle under vision with a stereoscopic zoom microscope (SMZ800, Nikon USA, Melville, New York). The LAD ligation was performed using an easily opened knot set on a PE50 silicon tube lying over the LAD. A total of 150 IU of heparin was injected intraperitoneally upon ligation to prevent microthrombosis. The ligation was released after 45 min followed by 1 h of reperfusion.

**Determination of MI size.** In those mice undergoing IR in which we sought to measure myocardial infarct size, 15 min before the end of the reperfusion interval, 0.5 cc of a 0.2% solution of propidium iodide (Sigma, Rehovot, Israel) was injected intraperitoneally. (Propidium iodide stains the nuclei of dead cells red when injected in vivo and, as discussed later, was used in this model to indicate infarcted myocardium.) At the end of the reperfusion interval, the LAD was re-occluded and a 4% solution of Thioflavin-S (Sigma) was injected into the ascending aorta. (Thioflavin stains endothelial cells blue when injected in vivo and was used in this model to indicate myocardium that was not at risk of MI upon LAD ligation.) The mice were then sacrificed and the left ventricle (LV) cryopreserved with liquid nitrogen-cooled methylbutane.

The LV was cut into 15-μm-thick cryosections, and every 20th section was photographed using an inverted fluorescent Zeiss microscope, connected to a digital camera and a computer with quantitative ImagePro software (Silver Spring, Maryland) (a total of 12 sections for each heart).

The area at risk of MI upon LAD ligation was defined and measured as thioflavin negative (i.e., the non-blue stained area). The infarct area was defined as propidium positive regions (i.e., deep red).

Quantitation of infarct size and risk area was performed using an infarct analysis program with Matlab software, using pixel color coordinates (color intensity) for automated calculation of the ratios: infarct area/risk area (IA/RA), infarct area/left ventricle (IA/LV), risk area/left ventricle (RA/LV). All quantitation was performed by a single reader blind to the Hp genotype of the preparations.

**Abbreviations and Acronyms**

**DM** = diabetes mellitus

**Hb** = hemoglobin

**Hp** = haptoglobin

**IA** = infarct area

**IL** = interleukin

**IR** = ischemia-reperfusion

**LAD** = left anterior descending coronary artery

**LPI** = labile plasma iron

**LV** = left ventricle/ventricular

**MI** = myocardial infarction

**RA** = risk area

**Measurement of lipid peroxidation products of arachidonic acid in myocardial tissue.** Lipid peroxidation products in myocardial tissue were measured both in mice completing the IR procedure as well as in uninstrumented mice. Hearts were rinsed 5 times in phosphate buffered saline containing antioxidant (0.1 mmol/l butylated hydroxytoluene) and metal chelator (2 mmol/l diethylenetriaminepentaacetic acid), transferred to screw-capped tubes containing butylated hydroxytoluene and diethylenetriaminepentaacetic acid, covered with argon atmosphere, and then snap frozen in liquid nitrogen and stored at −70°C until analysis.

Lipid extraction of the hearts was performed under either argon or nitrogen atmosphere as previously described (17). Multiple distinct oxidation products of arachidonic acid and linolenic acid were analyzed by reverse-phase high-performance liquid chromatography with online electrospray ionization tandem mass spectrometry (17).

**Measurement of redox active iron (LPI).** Heparinized plasma was collected from mice at the end of the IR interval and was stored at −70°C until assayed. Labile plasma iron (LPI) was measured as previously described using dihydrorhodamine, a sensitive fluorescent indicator of oxidative activity (18).

**Measurement of interleukin (IL)-10 in mice after IR.** Serum was collected at the end of the IR interval. Myocardial IL-10 was also measured in the myocardium of IR or control mice. Myocardial tissue was homogenized in phosphate-buffered saline with 1% triton and a protease inhibitor cocktail (Sigma Product #8340) and centrifuged at 140,000 g for 20 min, and IL-10 was measured in the supernatant. An enzyme-linked immunosorbent assay was used to measure IL-10 (BioLegent, Tel Aviv, Israel).

**Oral administration of an antioxidant.** BXT-51072 (19,20), a small molecular weight, orally bioavailable, catalytic mimic of glutathione peroxidase with potent lipid peroxidase activity, was obtained from Haptoguard (Fort
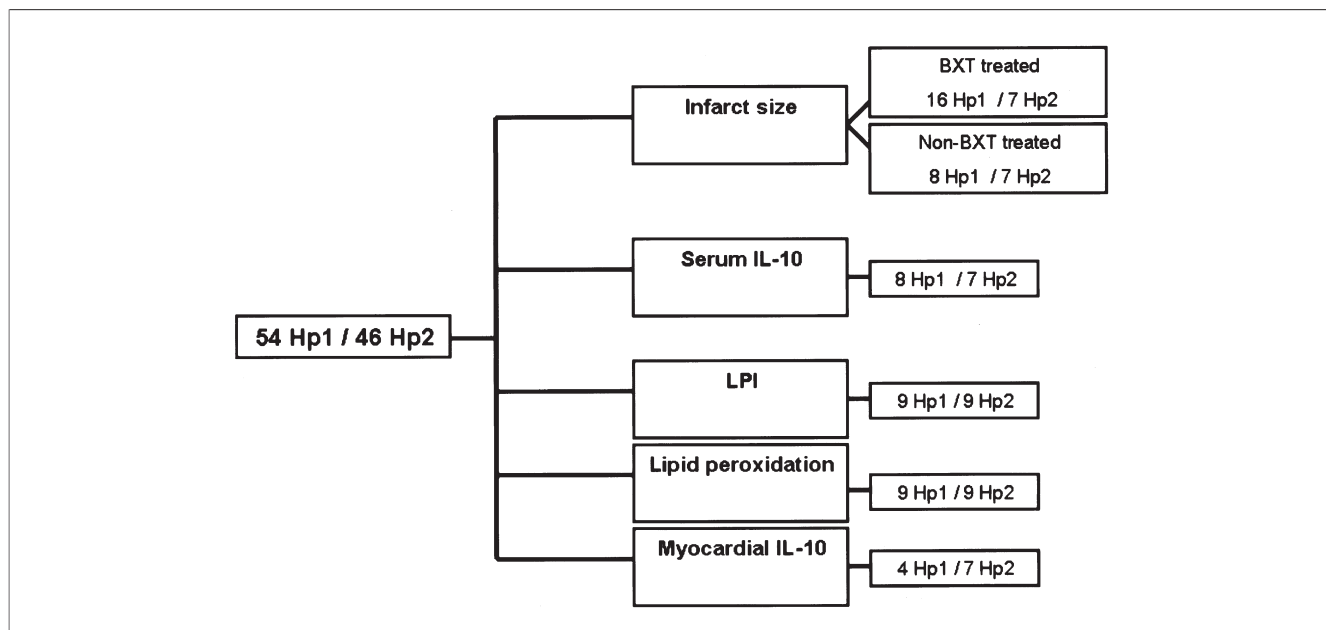
**Figure 1    Flow Diagram of Mice Completing the IR Protocol Used in This Study**

Fifty-four haptoglobin (Hp) 1 diabetes mellitus (DM) mice and 46 Hp 2 DM mice completing the ischemia-reperfusion (IR) protocol as described in the Methods section were used for measuring infarct size, cytokines, labile plasma iron (LPI), and lipid peroxidation. Infarct size was measured both in mice that did and did not receive BXT. BXT-treated mice received BXT before undergoing the IR protocol and non-BXT treated mice underwent the IR protocol but did not receive BXT. The number of mice used for each measurement is shown. In addition to the mice shown in the figure, lipid peroxidation was also measured in 5 Hp 1 and 5 Hp 2 mice that did not undergo the IR protocol. IL = interleukin.

Lee, New Jersey). BXT-51072 was prepared as a suspension in water at 1 mg/ml and was given by gastric lavage at a dose of 5 mg/kg (approximately 100 $\mu$l) 30 to 40 min before LAD ligation.

**Statistical analysis.** Groups were compared for the measured parameters using the Student $t$ test. All p values are 2-sided, and a p value of <0.05 was considered statistically significant. A Bonferroni adjustment was made for multiple comparisons.

### Results

**Experimental protocol flow chart.** The hearts and plasma from mice completing the IR protocol were analyzed for several parameters: MI size, lipid peroxidation products of arachadonic acid, redox active iron, and cytokines. All of these parameters could not be measured in the same heart/plasma from a given mouse because of differences in the way the heart/plasma needed to be prepared and the amount of material needed in order to measure these parameters. The flow chart in Figure 1

provides the number of Hp 1 and Hp 2 mice completing the IR protocol used in the measurement of each parameter. The size of the study groups used for all analyses was predetermined on the basis of anticipated differences between the groups as well as the known variation of each measurement. Mice were specifically set aside for gathering data for a given parameter. There was no difference in mortality between Hp 1 and Hp 2 mice undergoing the IR protocol.

**MI size is increased in Hp 2 mice.** We compared MI size in 7 Hp 2 DM mice and 8 Hp 1 DM mice subjected to IR. There were no significant differences in the age, duration of DM, or glucose or HbA1c levels between these Hp 1 and Hp 2 DM mice (Table 1). Furthermore, there was no significant difference in the area at risk of MI between these Hp 1 and Hp 2 mice (Table 2). However, there was a statistically significant increase in infarct size (IA/RA) in Hp 2 mice compared with Hp 1 mice (44.3 $\pm$ 9.3% vs. 21.0 $\pm$ 4.0%, n = 7 Hp 2 and n = 8 Hp 1 mice, p = 0.03) (Table 2).

| Table 1 | Baseline Characteristics of Mice Used to Determine Infarction Size | | | | | |
|---|---|---|---|---|---|---|
| Hp Genotype | n | Weight (g) | Age (months) | DM Duration (days) | Glucose (mg/dl) | HbA1c (%) |
| Hp 1 | 8 | 22.0 $\pm$ 1.30 | 4.3 $\pm$ 0.30 | 40.1 $\pm$ 1.5 | 417 $\pm$ 45 | 13.1 $\pm$ 0.8 |
| Hp 2 | 7 | 22.8 $\pm$ 0.70 | 4.2 $\pm$ 0.10 | 34.0 $\pm$ 3.6 | 388 $\pm$ 62 | 13.6 $\pm$ 0.6 |

All data are presented as the average $\pm$ SEM. Hemolglobin (Hb) A1c is expressed as the percentage of total Hb.
DM = diabetes mellitus; Hp = haptoglobin.

| Table 2 | Quantitation of Infarct Size in Hp 1 and Hp 2 Mice | | |
|---|---|---|---|
| **Hp Genotype** | **IA/RA (%)** | **IA/LV (%)** | **RA/LV (%)** |
| Hp 1 (n = 8) | 21.0 ± 4.0 | 16.0 ± 3.5 | 74.2 ± 6.7 |
| Hp 2 (n = 7) | 44.3 ± 9.3* | 27.0 ± 3.3† | 70.2 ± 9.0 |

All data are presented as mean ± SEM. *p = 0.03 and †p = 0.04 comparing Hp 1 and Hp 2 mice. Hp = haptoglobin; IA = area of myocardial infarction; LV = total left ventricular area; RA = area at risk of myocardial infraction with left anterior descending coronary artery occlusion.

**Marked increase in lipid peroxidation in the myocardium of Hp 2 mice after IR.** We measured a panel of lipid peroxidation products in the myocardium of non-instrumented (mice which were not subjected to the IR protocol) and IR treated DM Hp 1 and DM Hp 2 mice. We found no significant difference in lipid peroxidation products in DM Hp 1 (n = 5) and DM Hp 2 (n = 5) non-instrumented mice. However, after IR we found a highly significant increase in lipid peroxidation products in the myocardium of DM Hp 2 (n = 9) mice but not in DM Hp 1 (n = 9) mice (Table 3).

**Redox active iron (LPI) is increased in diabetic Hp 2 mice after IR.** We measured the amount of redox-active LPI after IR in Hp 1 and Hp 2 DM mice. We found that LPI was significantly elevated in Hp 2 mice as compared with Hp 1 mice (0.45 ± 0.11 $\mu$mol/l vs. 0.14 ± 0.05 $\mu$mol/l, n = 9, p = 0.02). We have previously demonstrated that LPI in non-instrumented Hp 1 and Hp 2 DM mice is markedly <0.1 $\mu$mol/l (9).

**IL-10 is markedly increased in Hp 1 mice after IR.** We found significantly higher serum levels of IL-10 in Hp 1 mice after IR as compared with Hp 2 mice after IR (85.8 ± 12.9 pg/$\mu$l vs. 46.7 ± 10.8 pg/$\mu$l, n = 8 Hp 1 and n = 7 Hp 2 mice, p = 0.04). There was no difference in IL-10 levels measured in myocardial homogenates of Hp 1 and Hp 2 mice subjected to IR (7.6 ± 1.1 pg IL-10/mg protein vs. 7.4 ± 0.8 pg IL-10/mg protein, n = 4 Hp 1 and n = 7 Hp 2 mice).

**Reduction in MI size with the glutathione peroxidase mimic BXT-51072 in Hp2 mice but not in Hp 1 mice.** We assessed the effect of the glutathione peroxidase mimic BXT-51072 (19,20) given by gastric lavage to Hp 1 or Hp 2 mice before IR. We found that BXT-51072 reduced MI size (IA/RA) in Hp 2 mice by more than 80% (44.3 ± 9.3% vs. 7.0 ± 3.1%, n = 7 for the non-BXT and BXT groups, p = 0.003). However, in Hp 1 mice, BXT did not reduce

MI size (21.0 ± 4.0% vs. 29.9 ± 6.5%, n = 8 for non-BXT and n = 16 for BXT group, p = 0.25).

## Discussion

In this study, we have demonstrated in a transgenic model that the amount of myocardial injury after IR is Hp genotype dependent. We have presented data supporting the hypothesis that this relationship between infarction size and Hp genotype is due to differences in the antioxidant and anti-inflammatory functions of the Hp 1 and Hp 2 allelic protein products. In Hp 2 mice, we have demonstrated an increased production of several lipid peroxidation products in the myocardium after IR. On the other hand, in Hp 1 mice, we have demonstrated an increased production of the anti-inflammatory and anti-oxidant cytokine IL-10 after IR.

Hemoglobin is a potent oxidant that is released from red cells because of hemolysis in the setting of IR and MI (21). We have proposed that the anti-oxidant and anti-inflammatory functions of Hp may be attributed to its interaction with Hb. We and others have demonstrated in vitro that the binding of Hp to Hb retards the ability of Hb to mediate lipid peroxidation and promotes the clearance of Hb via the monocyte/macrophage CD163 scavenging receptor (6–9). Furthermore, the binding of the Hp-Hb complex to CD163 promotes the release of anti-inflammatory cytokines (10,11). In vitro, in tissue culture, we have demonstrated that the Hp 1 protein is superior to the Hp 2 protein in these anti-oxidant and anti-inflammatory functions (6–11). The data presented herein, showing increased lipid peroxidation and increased redox-active iron in Hp 2 mice and increased IL-10 in Hp 1 mice, provide an in vivo validation of these in vitro observations.

We have demonstrated the importance of oxidative stress in the development of myocardial injury in Hp 2 mice after IR by showing that pharmacologic administration of an antioxidant dramatically reduces MI size in this model. We have not demonstrated that the antioxidant used in this study is superior to any other antioxidant that might have been given. However, the choice of supplementing the Hp 2 mice with a glutathione peroxidase mimic to test this hypothesis was not completely arbitrary. Glutathione peroxidase, an important defense mechanism against myocardial ischemia-reperfusion injury (22,23), is markedly decreased in the setting of DM (24–26). Moreover,

| Table 3 | Myocardial Lipid Peroxidation Products of Arachidonic Acid in Hp 1 and Hp 2 Mice With and Without IR | | | | | | |
|---|---|---|---|---|---|---|---|
| **Hp** | **PGF$_{2\alpha}$** | **5-HETE** | **8-HETE** | **9-HETE** | **11-HETE** | **12-HETE** | **15-HETE** |
| Hp 1 | 0.62 ± 0.09 | 0.32 ± 0.02 | 0.22 ± 0.01 | 0.32 ± 0.02 | 0.29 ± 0.01 | 1.41 ± 0.23 | 1.77 ± 0.16 |
| Hp 2 | 0.65 ± 0.05 | 0.35 ± 0.06 | 0.22 ± 0.01 | 0.32 ± 0.02 | 0.28 ± 0.01 | 1.12 ± 0.48 | 1.76 ± 0.17 |
| Hp 1 (IR) | 0.84 ± 0.07 | 0.41 ± 0.07 | 0.25 ± 0.05 | 0.37 ± 0.09 | 0.32 ± 0.07 | 2.23 ± 0.88 | 1.93 ± 0.34 |
| Hp 2 (IR) | 0.71 ± 0.20 | 0.39 ± 0.08 | 0.30 ± 0.03* | 0.42 ± 0.06* | 0.37 ± 0.04* | 2.85 ± 1.20* | 2.21 ± 0.31* |

All data are expressed as mean ± SEM in mmol/mol of the ratio of the lipid peroxidation product to arachidonic acid in the myocardium of non-instrumented (n = 5 for each genotype) and IR-treated mice (n = 9 for each genotype). *p < 0.05 (after adjustment for multiple comparisons) comparing Hp 2 (IR) mice to Hp 2 non-instrumented mice.
HETE = hydroxy-eicosatetraenoic acid; Hp = haptoglobin; IR = ischemia-reperfusion; PGF$_2$ = prostaglandin F$_2$.

glutathione peroxidase is the primary mechanism used by the red blood cell to protect its membrane from lipid peroxidation by the extraordinarily high Hb concentrations found within the red cell (27–29). In vitro and in vivo studies with BXT-51072 have shown that it is capable of protecting cells against reactive oxygen species, catabolizing lipid peroxides, and inhibiting inflammation presumably via its actions as a potent inhibitor of nuclear factor kappa beta (NF-KB) activation (19,20).

We did not anticipate that BXT-51072 would fail to prevent myocardial injury in Hp 1 mice, as overexpression of glutathione peroxidase has been demonstrated to decrease myocardial injury after IR in Hp 1 mice (22,23). This discrepancy may reflect differences in the levels of glutathione peroxidase activity in BXT-supplemented mice and glutathione peroxidase transgenic mice. One explanation for the failure of BXT to provide benefit to Hp 1 mice may be its ability to inhibit the induction of the myocardial protectant IL-10 normally induced in Hp 1 mice with IR (30). This blunting of the induction of IL-10 by BXT may be mediated by an inhibition of NF-KB activation (30,31). The use of antioxidants for the treatment of cardiovascular disease has fallen out of favor owing to the demonstration of a lack of benefit and possible harm associated with their administration (32,33). One hypothesis that has been put forth for the failure of these antioxidant trials to show benefit is that patient selection was not sufficiently selective. It has been proposed that antioxidant therapy may provide benefit only to a select population with markedly increased oxidative stress (34). Such a paradigm has been demonstrated in hemodialysis patients (35). The pharmacogenetic result presented here, wherein antioxidant therapy with BXT provided benefit to Hp 2 mice but not to Hp 1 mice, is consistent with this hypothesis. This result is all the more intriguing in light of our recent report that antioxidant therapy in the HOPE (Heart Outcomes Prevention Evaluation) study provided significant cardiovascular benefit to DM Hp 2-2 (homozygous for 2 allele) but not to DM individuals with the Hp 2-1 or Hp 1-1 genotypes (36).

This study has several limitations. First, although overall the total number of mice used was consistent with other transgenic studies using the IR model, a few of the measurements in this study relied on a small number of mice. Second, the Hp 2 allele does not normally exist in mice and may have resulted in physiologic changes that we have not measured. Third, it is not possible to know whether the associations that we have observed are specific to DM because we have not studied the described associations in these mice in the absence of DM. Finally, we have not directly demonstrated that the protective effects of BXT-51072 were mediated by its antioxidant action or by some other unknown mechanism.

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