Abstract—Hereditary hemochromatosis is an inherited pathological condition characterized by iron overload in several vital organs including heart. To increase our understanding of the underlying pathogenic mechanisms of hereditary hemochromatosis, we used a HFE gene knockout mouse model that replicates hereditary hemochromatosis. A group of mice with no copies of HFE gene and corresponding wild-type mice were maintained either on low-iron (30 ppm) or high-iron (300 ppm) diet since birth. The results of our study revealed that HFE gene knockout mouse hearts were susceptible to ischemia-reperfusion injury as evidenced by increased postischemic ventricular dysfunction, increased myocardial infarct size and cardiomyocyte apoptosis compared with wild-type control hearts. The degree of injury increased in the hearts of the mice fed high-iron diet. The hearts of the HFE knockout mice showed increased iron deposition, increased content of reactive oxygen species (ROS) as evidenced by the increased formation of malondialdehyde, and reduced antioxidant enzymes including superoxide dismutase, catalase, and glutathione peroxidase. The results suggest that increased amount of ROS and reduced antioxidant reserve secondary to iron overloading may be instrumental for the susceptibility of the HFE gene knockout mice to cardiac injury. (Circ Res. 2003;92:1240-1246.)

Key Words: reactive oxygen species ■ antioxidant enzymes ■ oxidative stress ■ iron overload ■ hereditary hemochromatosis

Hereditary hemochromatosis is an inherited pathological condition characterized by iron overload of parenchymal cells in several vital organs including heart due to increased iron absorption by the intestine, the primary site of regulation for iron homeostasis.1-2 A tight linkage of hereditary hemochromatosis with the HLA complex has long been recognized, but the underlying gene defect was only recently discovered with the identification of the HFE gene.3 The HFE gene has been found to be mutated in a large proportion of hereditary hemochromatosis patients.4 However, many homozygotes for the mutation do not manifest symptoms of iron-overload, suggesting that other genetic polymorphisms play a role, and that environmental conditions, such as dietary iron content, are also important to the pathological progression of hereditary hemochromatosis.

The iron overload in specific target organs leads to the development of cardiomyopathy, diabetes, arthritis, and cirrhosis of liver.5,6 Cardiac iron overload is likely to cause cardiac dysfunction leading to chronic cardiac failure. Indeed heart failure is a major cause of mortality among the untreated hereditary hemochromatosis patients and posttransfusional secondary hemochromatosis.7 The reason for this is not clear, but iron-catalyzed free radical generation and the level of antioxidant enzymes present in these patients may be instrumental for the heart failure resulting from hereditary hemochromatosis.

To increase our understanding of the underlying molecular mechanisms of myocardial damage in hereditary hemochromatosis, we used the HFE gene knockout mouse model that replicates the human condition.8

Materials and Methods

HFE Gene Knockout Mice
The HFE gene knockout mice were kindly supplied by Dr William S. Sly, St Louis University School of Medicine, St Louis, Mo. The knockout mice were developed by targeted disruption of the HFE gene.9 Northern blot analysis of HFE mRNA from the HFE+/− mouse hearts and wild-type hearts showed complete absence of the HFE gene in the HFE−/− mouse hearts. Four groups of mice were used for this study. HFE−/− and C57 wild-type mice were kept either on low-iron diet (30 ppm) or on high-iron diet (300 ppm) since birth, and experiments were performed with these mice after 3 months. The content of iron in the hearts was measured before experiment and at the end of the experiment.

Isolated Working Mouse Heart Preparation
All animals used in this study received humane care in compliance with the principles of laboratory animal care formulated by the...
National Society for Medical Research and Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (publication No. NIH 85-23, revised 1985). The mice were first anesthetized with sodium pentobarbital (200 mg/kg bw IP injection, Abbott Laboratories) and anticoagulated with Heparin (500 U/kg bw IP injection, Elkins-Sinn Inc). The heart was excised and immediately immersed in ice cold perfusion buffer. The aorta was cannulated and retrograde perfusion in the Langendorff mode through the aortic cannula was initiated at a perfusion pressure of 60 mm Hg. The perfusion buffer used in this study consisted of a modified Krebs-Henseleit Bicarbonate buffer [KHB: composed of (in mmol/L) 118 NaCl, 4.7 KCl, 1.2 MgSO4, 25 NaHCO3, 10 glucose, and 1.7 CaCl2, gassed with 95% O2:5% CO2, filtered through a 5-μm filter to remove any particulate contaminants, pH 7.4] that was maintained at a constant temperature of 37°C and was gassed continuously for the entire duration of the experiment. The method followed was essentially the same as that which has been described previously except for a slight modification in that for our model we used a fixed preload of 15 mm Hg and a fixed afterload of 50 mm Hg maintained by hydrostatic columns. After 10 minutes of retrograde perfusion, the heart was switched to antegrade perfusion mode where KHB buffer entered the cannulated left atrium at a pressure equivalent to 10 cm of water, and passed to the left ventricle from which it was spontaneously ejected through the aortic cannula against a pressure equivalent to 100 cm of water. Control measurements of heart rate, coronary flow, aortic flow, left ventricular end-diastolic pressure, left ventricular developed pressure, and its first derivative were recorded before ischemia. After baseline measurements, the antegrade perfusion line was closed, and the heart was subjected to 30 minutes of ischemia. All hearts were then reperfused for 2 hours in the working mode. Left ventricular, aortic, and left atrial pressures were monitored, analyzed, and recorded in real time using the Digitized data acquisition and analysis system (Micromed). Heart rate (HR), left ventricular developed pressure (DP) defined as the difference of the maximum and minimum left ventricular pressures, and maximum positive dLVP/dt were all derived or calculated from the continuously obtained left ventricular pressure signal. At the end of the 2-hour reperfusion period, hearts were removed from the apparatus and the atrial tissue was dissected away. The ventricles were either fixed in 10% buffered formalin or were immersed in 1% triphenyl tetrazolium chloride (TTC) solution in phosphate buffer (Na2HPO4 88 mmol/L, NaH2PO4 1.8 mmol/L) at 37°C for 10 minutes. Hearts kept in formalin were later embedded in paraffin following standard procedures and 3-μm thick transverse ventricular sections were obtained to perform TUNEL assays for the detection of apoptosis. Hearts immersed in TTC were stored at −70°C for later processing for infarct size.

Estimation of Iron, MDA Formation, and Antioxidant Enzymes in the Heart

The amount of nonheme iron in the hearts was analyzed after digestion in an acid mixture containing 3 mol/L HCl and 10% trichloroacetic acid for 24 hours at 65°C. About 300 μL of the acid extracts were mixed with 1.6 mL of bathophenanthroline chromogen reagent and the absorbance read at 535 nm with a Beckman DU-100000 spectrophotometer. MDA was estimated as described previously. The levels of the key antioxidant enzymes, superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHPx) were measured using standard enzymatic assay methods. In short, SOD was determined by its inhibitory action on the superoxide-dependent reduction of ferricytochrome by xanthine/xanthine oxidase by following the rate of cytochrome C reduction at 417 nm. Catalase was assayed by following the decomposition of H2O2 directly by the decrease in extinction at 240 nm. The assay of GSHPx-1 was based on the coupling of the enzyme to NADPH via GSH reductase, and the rate of NADPH oxidation was measured at 340 nm.

Assessment of Cell Death due to Necrosis and Apoptosis

Immunohistochemical detection of apoptotic cells was performed using TUNEL staining in conjunction with mouse monoclonal antibody recognizing cardiac myosin heavy chain followed by staining with TRIC-conjugated rabbit anti-mouse IgG (200:1 dilution) to specifically detect apoptotic cardiomyocytes. Myocardial infarct size was determined by standard TTC staining method using the NIH Image 1.6.1 image processing software system.

Statistical Analysis

The values for myocardial functional parameters, MDA, risk and infarct volumes, and infarct sizes were all expressed as the mean±SEM. For the statistical analysis, analysis of variance (ANOVA) followed by Scheffe’s test was first performed to test for any differences between the groups. If differences were established, differences between data were analyzed for significance by performing a Student’s t test. The results were considered significant if P<0.05.

Results

Iron Absorption in the Heart

As expected, hearts of the HFE−/− mice contained increased levels of iron compared with those of the wild-type heart (Figure 1). The wild-type mouse fed high-iron diet had relatively, but not significantly, higher amount of iron in the heart. Significantly higher amount of iron was found in the HFE−/− mouse hearts fed both low and high-iron diet. The iron content of the heart increased dramatically in the hearts of mice fed diet containing 300 ppm iron (Figure 1, left). The amount of the iron content in the ischemic-reperfusion hearts did not vary significantly compared with those found at the baseline levels.

Reactive Oxygen Species in the Heart

The production of MDA is an index of the occurrence of lipid peroxidation and the development of oxidative stress. At baseline, the MDA level of the hearts of wild-type mice was minimal; the mice fed 300 ppm iron diet being slightly, but not significantly higher (Figure 1, right). At the end of 30 minutes of ischemia and 2 hours of reperfusion, MDA content of the wild-type mouse hearts increased significantly in both groups of wild-type hearts compared with baseline values. A dramatic increase in MDA content was found in the HFE knockout mouse hearts, both at the baseline levels and at the end of ischemia and reperfusion. Thus, about 5-fold increase in MDA content was found in the HFE knockout mouse hearts subjected to ischemia/reperfusion as compared with its baseline values.

Levels of Antioxidant Enzymes in the Hearts of HFE−/− and Wild-Type Mice

Because antioxidant enzymes comprise the primary defense line of the hearts, we measured the key antioxidant enzymes, SOD, catalase, and GSHPx-1, in the hearts at baseline and at the end of ischemia/reperfusion. As shown in Figure 2, Mn-SOD and GSHPx-1 levels, but not Cu/Zn-SOD and catalase levels, were lower in the HFE knockout mouse hearts fed high-iron diet at the baseline level. At the end of ischemia/reperfusion, levels of all the antioxidant enzymes tested, except for Cu/Zn-SOD, which showed lowering in
activities only for high-iron diet–fed hearts, were lowered compared with wild-type controls. This is not unexpected: ischemia/reperfusion is known to reduce intracellular antioxidant reserve in the myocardium. Interestingly, for wild-type control, GSHPx-1 activity was also lower in the hearts fed high-iron diet.

Recovery of Myocardial Contractile Performance

Because of the high incidence of ventricle fibrillation and ventricle arrhythmia, two hearts each were excluded from HFE<sup>+/−</sup> 30-ppm and HFE<sup>+/−</sup> 300-ppm groups. We analyzed 8 hearts for ventricular function from each group without showing any sign of the cardiac conductive disturbance throughout the experimental period. There were no statistically significant differences between the wild-type and the knockout groups with regard to baseline cardiac parameters.

Evidence of impaired recovery cardiac function was clearly apparent in HFE<sup>+/−</sup> mice from as early as R15', although statistically significant difference could be detected only from R30' onward (Figures 3 through 5). The left ventricle develop pressure was dramatically decreased in those HFE<sup>+/−</sup> hearts to be given high-iron supplemented diet (300 ppm) (Figure 3, top). This tendency was observed at R15' (63.40±2.77 versus 69.73±4.0 mm Hg in the control group), R30' (51.68±1.88 versus 63.18±3.44 mm Hg in control group), R60' (42.13±1.55 versus 54.13±2.64 mm Hg in control group), and R120' (34.25±1.25 versus 45.50±1.72 mm Hg in control group). Only the 30,120 minutes of left ventricle function of HFE<sup>+/−</sup> animals with 30-ppm iron showed statistical differences for their correspondent control group’s cardiac parameters.

The left ventricular end-diastolic pressure of the HFE<sup>+/−</sup> animals fed with 300-ppm iron-containing food displayed elevation of pressure at R30' (14.13±0.72 versus 11.77±0.43 mm Hg), R60' (17.66±0.67 versus 13.28±0.32 mm Hg), and R120' (20.53±0.97 mm Hg).

Figure 1. Left, iron content of the heart. Wild-type and HFE knockout mice were maintained on low- (30 ppm) and high- (300) iron diet. Isolated hearts were made globally ischemic for 30 minutes followed by 2 hours of reperfusion. Iron content was estimated at baseline and at the end of ischemia and reperfusion. Right, MDA content of the heart at baseline and at the end of reperfusion. Results are shown as mean±SEM of 6 animals per group.

Figure 2. Effects of low- and high-iron diet on the intracellular antioxidant enzymes of the heart. Myocardial content of Mn- and Cu/Zn-SOD, catalase, and GSHPx-1 are shown at baseline and at the end of reperfusion: , wild-type; , HFE knockout. Results are shown as mean±SEM of 6 animals per group. *P<0.05 vs wild-type; †P<0.05 vs 30 ppm.
versus 14.27±0.40 mm Hg) (Figure 3, middle). The HFE<sup>−/−</sup> hearts with 30-ppm caused also higher significant LVEDP values at 60,120 time point of recovery period compared to wild-type heart function. Dp/Dt values representing the maximum first derivative of left ventricle develop pressure showed significant reduction in both of HFE<sup>−/−</sup> groups at each time point of reperfusion period as compared with wild-type controls (Figure 3, bottom).

Heart rates remained unchanged and preserved close to the baseline values for both HFE<sup>−/−</sup> and wild-type animals for the entire duration of experimental period (Figure 4, top). The cardiac output, which is the main cardiac function, exhibited similar postischemic recovery pattern (Figure 4, bottom). The aortic flow displayed gradual depression throughout the reperfusion period as compared with baseline values (Figure 5, top). HFE<sup>−/−</sup> 300-ppm group indicated significant impairment compared with wild-type group R30<sup>−/−</sup> 0.30±0.02 versus 0.47±0.04 mL/min), R60<sup>−/−</sup> 0.2±0.02 versus 0.30±0.02 mL/min), and R120<sup>−/−</sup> 0.13±0.01 versus 0.23±0.03 mL/min). The knockout mice fed low-iron diet also exhibited statistical difference to wild-type controls at 30,60 minutes of reperfusion time. The coronary flow showed the same pattern as the aortic flow at each point of reperfusion related to control value with exception of R30<sup>−/−</sup> of HFE<sup>−/−</sup> 30-ppm heart function (Figure 5, bottom).

Infarct size, expressed as the ratio of percent infarction to total area at risk, was noticeably increased in the knockout vs wild-type.
mouse heart transverse sections compared with the wild-type control sections (44.65 ± 1.37% of HFE−/− 300-ppm, 38.90 ± 1.93% of HFE−/− 30-ppm versus 32.75 ± 1.85% of wild type 300-ppm, 30.00 ± 1.45% of wild type 30-ppm) (Figure 6, top). Significant difference was observed between wild-type 300-ppm and HFE−/− 30 ppm and HFE−/− 300-ppm heart cross-sections compared with wild-type 30 ppm iron.

**Apoptotic Cell Death**

As shown in Figure 6, bottom, both wild-type and HFE−/− hearts displayed significant number of apoptotic cardiomyocytes after 30 minutes of ischemia and 2 hours of reperfusion. We used double antibody to specifically stain cardiomyocytes. Thus, in conjunction with TUNEL, we used monoclonal antibody recognizing cardiac myosin heavy chain followed by staining with TRIC-conjugated rabbit anti-mouse IgG. The results shown in Figure 6, bottom, indicate significantly larger number of apoptotic cardiomyocytes in the HFE−/− mouse hearts compared with corresponding wild-type hearts. The number of apoptotic cells was higher for the hearts fed diet containing high iron.

**Discussion**

In these experiments, we found that iron content of the diet interacted with the HFE polymorphism to increase ischemia/reperfusion injury to the heart. There was a remarkable “dose-response” shown in increasing infarct size, increased percentage cardiomyocyte apoptosis, and reduced ventricular recovery when examined in the order of low-iron diet wild-type mice, high-iron wild type, low-iron HFE, and high-iron HFE. This order for myocardial injury mirrored the non-heme iron content of the hearts of the animals, the lowest heart iron content being in the low-iron diet wild-type mice. In addition, levels of antioxidant enzymes were lowered in the same order, and among the HFE−/− mice on the high-iron diet, we observed significantly greater MDA concentration in heart muscle than in the HFE−/− on the low-iron diet. These results are consistent with a direct effect of iron on myocardial injury mediated by generation of free radicals. Thus, elevated body iron may be a risk for both heart disease and cancer by a similar free-radical mechanism, and the HFE polymorphism may represent a susceptibility to injury depending on the iron content of the diet.

Iron overload is the most common metal-related toxicity that can result in cardiomyopathy and arrhythmias leading to heart failure. Iron-overload cardiomyopathy is characterized by the increased deposition of iron in the myocardium leading to systolic or diastolic cardiac dysfunction. The exact mechanism(s) by which iron overload induces myocardial injury is not completely understood, but the results demonstrate the presence of increased granules of both Fe<sup>2+</sup> and Fe<sup>3+</sup> in the hearts of the chronically iron-loaded mice. These mouse hearts showed dramatic alterations to heart architecture, including evidence for a decrease in the abundance of myofibril elements and mitochondria. Interestingly, these hearts also showed reduced amount of GSHPx activity and increased amount of aldehydes, suggesting increased development of oxidative stress in these hearts. It is believed that reactive oxygen species (ROS) may play a role in iron-overload-cardiomyopathy and heart failure. For example, electron paramagnetic resonance (EPR) spectroscopy revealed the massive generation of hydroxyl radicals (OH·) in the iron-overloaded hearts as well as in the ischemic reperfused myocardium. However, the mechanism of the role of iron in increased ROS production in the ischemic reperfused myocardium may be quite different from that for the chronically iron-overloaded hearts. In the ischemic reperfused myocardium, ROS result in abnormal ventricular function giving rise to “stunning”, whereas the chronically iron-loaded hearts become extremely susceptible to infarction. It is tempting to speculate that in cases of chronically iron-loaded hearts, iron is bound in ferritin store and free iron may not be available for OH· radical formation during ischemia/reperfusion. The iron-loaded hearts are, on the other hand, exposed to increased oxidative stress because of the presence of reduced amount of antioxidant reserve and increased amount of aldehydes, suggesting increased oxidative stress in these hearts. It is believed that reactive oxygen species (ROS) may play a role in iron-overload-cardiomyopathy and heart failure. For example, electron paramagnetic resonance (EPR) spectroscopy revealed the massive generation of hydroxyl radicals (OH·) in the iron-overloaded hearts as well as in the ischemic reperfused myocardium. However, the mechanism of the role of iron in increased ROS production in the ischemic reperfused myocardium may be quite different from that for the chronically iron-overloaded hearts. In the ischemic reperfused myocardium, ROS result in abnormal ventricular function giving rise to “stunning”, whereas the chronically iron-loaded hearts become extremely susceptible to infarction. It is tempting to speculate that in cases of chronically iron-loaded hearts, iron is bound in ferritin store and free iron may not be available for OH· radical formation during ischemia/reperfusion. The iron-loaded hearts are, on the other hand, exposed to increased oxidative stress because of the presence of reduced amount of antioxidant reserve and increased amount of aldehydes, suggesting increased oxidative stress in these hearts. It is believed that reactive oxygen species (ROS) may play a role in iron-overload-cardiomyopathy and heart failure. For example, electron paramagnetic resonance (EPR) spectroscopy revealed the massive generation of hydroxyl radicals (OH·) in the iron-overloaded hearts as well as in the ischemic reperfused myocardium. However, the mechanism of the role of iron in increased ROS production in the ischemic reperfused myocardium may be quite different from that for the chronically iron-overloaded hearts. In the ischemic reperfused myocardium, ROS result in abnormal ventricular function giving rise to “stunning”, whereas the chronically iron-loaded hearts become extremely susceptible to infarction.
hemochromatosis patients. The serum from the β-thalassemia major patients also contains lower amounts of antioxidants and higher levels of lipid peroxidation products in their serums. These results support the notion that iron-catalyzed OH· radical formation (Fenton Reaction) may be primarily responsible for the formation of ROS, which then lead to cellular injury. Indeed, the results of our study document that the amount of MDA content and lower amounts of antioxidant enzymes. The results thus confirm that HFE knockout mouse hearts.

A wide variety of coronary heart diseases have been linked with the overproduction of oxygen-derived free radicals. The results of many studies, including our own, have demonstrated that this excessive production of ROS in concert with drastic reduction of antioxidant reserve play a crucial role in the pathophysiology of ischemic heart disease. Myocardial ischemia and reperfusion cause the cardiomyocytes to face conditions that shift their redox status to undergo a drastic change subjecting them to oxidative stress. Interventions with oxygen free radical scavengers or antioxidant therapy have been found to be cardioprotective against ischemic reperfusion injury. Recent studies have documented that coronary heart disease cause cardiomyocyte death not only by necrosis but also by apoptosis. Reperfusion of ischemic myocardial results in apoptotic cell death and DNA fragmentation, and antioxidants such as Ebselen, a glutathione peroxidase mimic, could reduce cardiomyocyte apoptosis. The hearts from the transgenic mice overexpressing GSHPx-1 gene were resistant to ischemia/reperfusion injury and cardiomyocyte apoptosis, whereas those from the GSHPx-1 knockout animals, devoid of any copy of GSHPx-1, were extremely vulnerable to the cellular injury as compared with wild-type controls. The results of the present study show that HFE−/− mouse hearts are subjected to excessive oxidative stress and greater degree of ischemic reperfusion injury as evidenced by increased myocardial infarct size and cardiomyocyte apoptosis and reduced postischemic ventricular recovery. The role of ROS is further supported from the results that HFE−/− hearts of the mice fed high-iron diet were subjected to greater degree of oxidative stress and as a result greater degree of myocardial injury.

Mammalian hearts are protected from the cellular injury by their own defense system, which includes various intracellular antioxidants, such as glutathione, α-tocopherol, ascorbic acid, β-carotene; and antioxidant enzymes that include SOD, catalase, and glutathione peroxidase. These cellular compounds reduce/eliminate the oxidative stress by directly quenching the reactive oxygen species before they damage vital cellular components, and therefore, they can be considered as part of the first line of defense against the external stress. Often, because of the inadequacy of the intracellular antioxidants or due to the presence of increased amount of oxidative stress, the reactive oxygen species may reach their targets that include nucleic acids, proteins, and lipids resulting in DNA strand breaks, protein degradation, and lipid peroxidation. The hearts of the HFE−/− mice had reduced levels of SOD, catalase, and GSHPx compared with those present in the wild-type controls. The levels of these antioxidant enzymes reduced dramatically in the hearts of the HFE−/− mice fed high-iron diet, suggesting that increased iron levels resulting in a higher amount of oxidative stress could be instrumental for the reduced level of antioxidant enzymes in these hearts.

Given the fact that HFE mutation is common in certain ethnic portions of the population, that about 10% or these populations are heterozygotes, and that heterozygotes exhibit higher body iron levels than normal, the interaction of diet and the polymorphism has a potentially large impact on health of the population. Both experimental and epidemiological findings support the hypothesis that lowering iron availability can decrease risk of coronary events. The importance of iron in the pathogenesis of myocardial ischemia/reperfusion injury and heart failure is well established. Epidemiological investigation also supports the role of increased iron in long-term risk of coronary events, including myocardial infarction. Further work should include examination of possible increased susceptibility of heart to other insults, and examination of other genetic polymorphisms that affect or modify the HFE gene in iron absorption. Future experiments should also investigate the impact of antioxidant administration on the ability of the high-iron diet to induce cardiac damage, as well as consideration of other dietary constituents that may modify the absorption and/or availability of iron such as phytate and vitamin C.

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Tibor Turoczi, Li Jun, Gerald Cordis, James E. Morris, Nilanjana Maulik, Richard G. Stevens and Dipak K. Das

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