Decreased Sodium and Increased Transient Outward Potassium Currents in Iron-Loaded Cardiac Myocytes
Implications for the Arrhythmogenesis of Human Siderotic Heart Disease

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Background—Patients with chronic iron overload may develop a cardiomyopathy manifested by ventricular arrhythmias and heart failure. We hypothesized that iron-loaded cardiomyocytes may have abnormal excitability.

Methods and Results—We examined a new model of human iron overload, the Mongolian gerbil given repeated injections of iron dextran. In ventricular myocytes, we measured iron concentration and distribution, action potential, sodium and potassium currents, and sodium channel protein. We showed for the first time that (1) the iron content of gerbil ventricular cardiomyocytes was increased to amounts similar to those of patients with iron-induced cardiomyopathy; (2) the overshoot and duration of the cardiac action potential decreased; (3) sodium current was reduced, steady-state inactivation was enhanced, and single-channel currents were unchanged; and (4) transient outward potassium current was increased, but inwardly rectifying potassium current was unchanged. Neonatal rat cardiomyocytes incubated with iron for 1 to 3 days showed similar changes, and levels of cardiac sodium channel proteins were unchanged.

Conclusions—Abnormal excitability and heterogeneous cardiac iron deposition may cause the arrhythmogenesis of human siderotic heart disease. (Circulation. 1999;100:675-683.)

Key Words: iron overload • cardiomyopathy • gerbil • sodium channels • potassium channels

Iron-overload cardiomyopathy produces arrhythmias1,2 and heart failure.1–4 It is the most frequent cause of death in thalassemia major,1,3,4 and a life-limiting complication of transfusion-dependent refractory anemias, hereditary hemochromatosis, and other forms of iron overload.2 Because iron excretion in humans is deficient, iron contained within transfused red cells or absorbed from the diet in excess of requirements progressively accumulates within the body.5 Eventually, a characteristic pattern of heterogeneous iron deposition develops within the heart, with the greatest amounts in the left side of the ventricular septum and free wall (especially in the epicardium), smaller amounts in the right ventricle, and still less in the atria.6,7 Within the myocardium, the iron is found almost entirely within myocardial cells and not within the interstitium. Past efforts to examine the effects of iron on the heart have been hampered by the lack of a suitable experimental model of human iron overload; most animal species have a far greater capacity to excrete iron than humans. Recently, Carthew et al8,9 reported that weekly subcutaneous injections of iron dextran to Mongolian gerbils reproduced critical features of the cardiomyopathy found in human iron overload, in part because the gerbil seems unable to excrete iron as effectively as other rodents. We hypothesized that abnormal excitability of iron-loaded heart cells might contribute to arrhythmias, and we examined membrane currents of single cardiomyocytes isolated from the hearts of iron-loaded gerbils. For comparison with a related, more extensively studied species, we used an in vitro model of cultured neonatal rat cardiomyocytes that were incubated with iron for several days.10,11

Methods

Nonheme Iron Concentrations

Nonheme iron concentrations of heart tissue and cardiomyocytes were determined as previously described.12

Electron Microscopy

Cardiomyocytes were prepared according to the method of Iancu et al13 and examined in a CEM 902 electron microscope (Carl Zeiss Inc.).

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Cultures of Neonatal Rat Cardiomyocytes

One-day-old rats (Sprague-Dawley; Zivic-Miller Laboratories, Portersville, PA) were anesthetized with Nembutal (50 mg/kg body weight), and hearts were removed surgically. Ventricular cardiomyocytes were isolated using the Neonatal Cardiomyocyte Isolation System.\textsuperscript{14} Cells were plated at 2.5×10⁶ cells/cm² onto 35- or 100-mm culture dishes (Falcon) in Dulbecco’s modified Eagle medium/nutrient mixture F-12 (1:1) (GIBCO) supplemented with 10% calf serum and 50 μg/mL gentamicin, and then they were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. After incubation for 16 to 18 hours, cells were washed twice with serum-free Dulbecco’s modified Eagle F-12 medium, and ferric ammonium citrate was added at a final concentration of 40 or 80 μg of elemental iron/mL. Both test and control cells were then incubated for 24 to 72 hours, and the medium was changed every 24 hours. All procedures conformed to institutional guidelines for the care and use of animals in research.

Isolation of Gerbil Cardiomyocytes

Mongolian gerbils (females, 6 to 8 weeks old; from Charles River Laboratories, Portage, Mich) were given subcutaneous injections of iron dextran at 200 mg Fe · kg⁻¹ · wk⁻¹. Hearts were prepared using Langendorff perfusion and the modified enzymatic method.\textsuperscript{15,16} After perfusion, the left ventricular epicardium was excised, minced, and gently shaken. Dissociated myocytes were stored for 2 to 24 hours at 4°C in a solution containing (in mmol/L): KCl 85, K₂HPO₄ 30, MgCl₂ 5, creatine 5, taurine 20, glucose 20, EGTA 0.125, β-OH-butyric acid 5, Na₃ATP 2, pyruvic acid 5, and CaCl₂ 0.02 and 50 g/L PVP-40 (pH 7.2). Quiescent, rod-shaped cells were selected for electrophysiology.

Electrophysiology

The cardiac action potential (CAP) of gerbil myocytes was recorded using the whole-cell gigaseal technique.\textsuperscript{17} Pipette and bath solutions were, respectively, (in mmol/L): potassium aspartate 140, MgCl₂ 5, HEPES 10, EGTA 10, glucose 10, and Na₂ATP 2 (pH 7.4). To zero membrane potential, the bath solution contained (in mmol/L): KCl 8, potassium aspartate 25, sodium aspartate 5, MgCl₂ 3, and HEPES 10 (pH 7.2). The measured liquid junction potential (LJP) was −2 mV. CAP from cultured neonatal rat myocytes was recorded using the perforated patch method with amphotericin B.\textsuperscript{18} The pipette solution was, respectively, (in mmol/L): KCl 8, potassium aspartate 25, sodium aspartate 5, K₂SO₄ 60, MgCl₂ 1, HEPES 10, EGTA 1, glucose 10, and sucrose 20 (pH 7.2). The measured LJP was −7 mV. CAPs were evoked by depolarizing current pulses (150 pA and 5 ms for gerbil cells or 10 pA and 2 ms for neonatal rat cells) at 1 Hz, with 30-s intervals between series. The first CAP in each series was used to compare parameters, and we always corrected for LJP.

For neonatal rat Na⁺ currents, the pipette and bath solutions were, respectively, (in mmol/L): CsCl 120, NaCl 10, CaCl₂ 1, MgCl₂ 2, EGTA 11, HEPES 10, and Na₃ATP 2 (pH 7.2) and NaCl 140, CsCl 10, KCl 5, MgCl₂ 5, CaCl₂ 0.02, 4-aminopyridine 5, HEPES 10, and glucose 10 (pH 7.4). External Ca²⁺ was replaced to minimize Ca²⁺ currents. Gerbil Na⁺ current was much larger, so we reduced the Na⁺ concentration. For gerbil Na⁺ currents, the pipette and bath solutions were, respectively, (in mmol/L): CsCl 120, CaCl₂ 1, MgCl₂ 2, EGTA 11, HEPES 10, and Na₃ATP 2 (pH 7.2) and NaCl 140, CsCl 10, KCl 5, MgCl₂ 5, CaCl₂ 0.02, 4-aminopyridine 5, HEPES 10, and glucose 10 (pH 7.4). External Ca²⁺ was reduced to minimize Ca²⁺ currents. Gerbil Na⁺ current was much larger, so we reduced the Na⁺ concentration. For gerbil Na⁺ currents, the pipette and bath solutions were, respectively, (in mmol/L): CsCl 120, CaCl₂ 1, MgCl₂ 2, EGTA 11, HEPES 10, and Na₃ATP 2 (pH 7.2) and NaCl 20, N-methyl-d-glucamine 75, aspartate 75, CsCl 20, KCl 5, MgCl₂ 5, CaCl₂ 0.02, 4-aminopyridine 5, HEPES 10, and glucose 10 (pH 7.4). The series resistance was 3 to 4 megohms, and 90% series resistance compensation was used. Records were low-pass filtered at 1 kHz and sampled at 2 kHz. Data were corrected for a LJP of −10 mV.

Data acquisition and analyses were performed with an Axopatch-1D amplifier, TL-1 interface, and pClamp 5.5.1 or 6 software (Axon Instruments). All experiments were performed at room temperature (20°C to 23°C).

Western Blot Analysis

Equal amounts of total membrane protein (~5 μg) isolated from control and iron-treated cardiomyocytes were separated on a 7.5% SDS-PAGE gel and transferred to a nitrocellulose membrane using an electrical field of 45 V for 16 hours at 4°C. The membrane was probed with the sodium-channel rH1 subtype–specific polyclonal antibody D-492.\textsuperscript{20} Signals were detected using anti-rabbit secondary antibody followed by electrogenerated chemiluminescence (Amer sham Life Sciences Inc) detection methods.

Statistics

The Student’s  test was used to compare 2 population samples, and ANOVA was used for >2.
Iron Loading

In the left ventricle and liver from gerbils loaded with iron by weekly injections of iron dextran, the iron level increased progressively (Figure 1), whereas control gerbils were unchanged. In whole hearts from gerbils loaded with iron for 20 weeks, the concentration increased almost 10-fold above controls (from 0.3 ± 0.1 mg of Fe/g of heart, dry weight, to 2.9 ± 1.6 mg of Fe/g of heart; n = 5). For comparison, arrhythmias and heart failure develop in patients with left ventricular nonheme iron concentrations of ≥2.0 mg of Fe/g heart, dry weight. In cultured neonatal rat cardiomyocytes incubated with 80 μg/mL Fe for 3 days, the increase was also ~10-fold (from 0.7 ± 0.2 mg of Fe/g of cells, dry weight, to 8.7 ± 0.9 mg of Fe/g of cells; n = 4). In control cells and during incubation with iron, the cell density remained constant. Iron loading also produced cytological changes. Iron was present only in loaded neonatal rat cardiomyocytes (Figure 2), and it appeared as electron-dense granules scattered throughout the cytoplasm or deposited within membrane-bound cytoplasmic organelles known as siderosomes, as reported previously. In control gerbils, 100 myocytes were examined, and a total of 8 siderosomes, averaging 0.7×0.6 μm, were observed. In 2

Figure 2. Representative electron micrographs of rat neonatal cardiomyocytes after 72 hours of incubation in control medium (A) or 80 μg/mL Fe (B) and gerbil cardiomyocytes taken from control left ventricular epicardium (C) or after weekly injections of iron dextran for 8 weeks (D). Cytosol of control rat cardiomyocytes (A) contains no visible electron-dense particles. In iron-loaded cardiomyocytes (B), membrane-bound bodies (siderosomes; arrows) contain many electron-dense ferritin particles. Some cardiomyocytes from control gerbil (C) contain single siderosomes (arrow). In cardiomyocytes from iron-loaded gerbil (D), number of siderosomes is increased. Note that cytosol of gerbil cardiomyocytes contains visible, electron-dense ferritin particles (C and D; arrowheads). Unstained. Bars=0.5 μm.
iron-loaded gerbils (8 weeks), 100 myocytes contained 39 siderosomes, averaging 3.2×2.0 μm.

**Cardiac Action Potential**

In rats, iron loading significantly reduced the overshoot of CAP (from 50.7±2.8 mV [n=6] to 25.5±4.8 mV [n=10]; P<0.002) and shortened the duration (action potential duration at 50% repolarization) from 263.3±44.3 to 108.2±45.3 ms; P<0.038) without significant change in resting membrane potential (−68.1±0.4 and −67.2±0.6 mV, respectively). The results were consistent with earlier reports of this model that used monolayers rather than single cells. In gerbils, data were pooled from 3 control and 3 iron-loaded animals. The overshoot was reduced (from 27.5±9.0 mV [n=7] to −1.5±6.4 mV [n=10]; P<0.02). The resting membrane potential was depolarized (from −71.8±0.3 to −67.5±0.9 mV; P<0.02) but, despite depolarization, the action potential duration at 50% repolarization was shortened (from 6.3±1.4 to 5.2±0.6 ms) (Figure 3A). In iron-loaded cells, the overshoot increased with repetitive pulsing, perhaps related to the reduced availability of the transient outward K+ current (Ito). In both rat and gerbil cells, all electrophysiological measurements were recorded in the absence of iron in the extracellular solution and were not influenced by the addition of iron as ferric ammonium citrate at 80 mg/mL. Control values of resting potential and action potential durations for neonatal rat cells are consistent with those from reports using similar conditions. Likewise, the control resting potential and action potential durations of gerbil cardiomyocytes are similar to values reported for adult rats.

**Sodium Currents**

Iron overload significantly reduced Na+ currents in both gerbil and rat models (Figures 3B and 4A). The measure-
ments in control and iron-loaded myocytes were made at equally brief times <5 minutes after penetration using pipettes with similar access resistances. In this way, time-dependent, hyperpolarizing shifts in inactivation were minimized and kept similar among the different groups of cells. Under these conditions, no differences existed in the voltage-dependence of the peak currents between control and iron-loaded cells, and the current-voltage relationships were simply scaled (Figures 3C and 4B). For rat cells, it was possible to show that the effects of incubation with iron were concentration-dependent and cumulative with time. At 80 μg/mL Fe, currents were unaffected on day 1, reduced by day 2, and significantly reduced by day 3 (P<0.01, Figure 4E). At 40 μg/mL Fe, the depression on day 3 was still statistically significant, but it was about half of that produced by the 80 μg/mL Fe dose.

The time course of sodium current activation and inactivation was unchanged. At steady-state, the activation-voltage relationships were similar, but inactivation shifted significantly toward hyperpolarized potentials in both gerbil and rat cells (Figures 3D and 4C). Recovery from inactivation at −140 mV was slowed significantly in both species: in gerbils, from 3.39±0.24 ms (n=7) to 6.83±1.48 ms (n=4; P<0.013; Figure 3E) and in rats, from 2.06±0.26 ms (n=5) to 3.84±0.66 ms (n=5; P<0.044; Figure 4D).

Experiments on cell-attached macropatches from rat cardiomyocytes loaded with the higher dose of iron for 72 hours gave results consistent with whole-cell experiments. Currents were significantly reduced by iron, and steady-state inactivation was shifted toward hyperpolarized potentials [voltage of half-maximal inactivation (V_{1/2})] −78.6±3.2 mV [n=3] to −88.9±4.8 mV [n=4]; P<0.05; slope factors, 5.6±0.75 and 4.8±1.3 mV] (Figure 5A).
Single-channel events at suprathreshold negative potentials (−60 and −70 mV) were sufficient for current and open-time histograms to be collected and analyzed. Single-channel conductance was 13.8±1.9 pS (n=4) and 14.6±1.5 pS (n=4) in control and iron-loaded cells, respectively. The mean open times were 0.34±0.03 ms (n=4) and 0.41±0.07 ms (n=4) for control and iron-loaded cells, respectively; they agreed with values reported by others at similar potentials in isolated neonatal rat cardiomyocytes.23 The opening probability was reduced in iron-loaded cells, and this was reflected in the averaged single-channel currents (Figure 5C). At a test potential of −10 mV (Figure 5B), the average current per patch was 47±13 pA (n=5) and 15±6 pA (n=9) for control and iron-loaded cells, respectively, which is consistent with the results at −60 mV (Figure 5C).

**Sodium Channel Protein**

To test whether Na⁺ channel protein was changed, we analyzed Western blots on rat cardiomyocytes incubated with 80 μg/mL Fe for 3 days. We found that the amount of Na⁺ channel protein was similar between control and iron-treated cardiomyocytes (Figure 6A) and, on average, the latter showed an increase of 22±7%, which was not statistically significant (Figure 6B).

**Potassium Currents**

Representative K⁺ currents from control and iron-loaded gerbil cardiomyocytes are shown in Figure 7A. Iron loading had no effects on either the non-inactivating K⁺ outward current (Iₜₒ) or the inward K⁺ current (Iₖᵢ) (Figure 7B) measured at 400 ms. In contrast, peak current (Iₚₑ𝐚𝓽) was significantly increased (Figure 7B). Iₚₑ𝐚𝓽 was measured at positive potentials to exclude contamination with sodium current (Iₙ𝓪ᵃ). Similar results were obtained in cultured rat myocytes (not illustrated). Time constants for Iₜₒ at 50 mV were 57.7±1.9 ms (n=6) and 52.8±2.6 ms (n=6) for control and iron-loaded myocytes, respectively. Steady-state inactivation shifted positively ≈5 mV (Figure 7C), but recovery from inactivation at −100 mV was not affected (Figure 7D). The voltage-dependence of steady-state inactivation and recovery of Iₜₒ in gerbils are different from values reported for adult rats24 but similar to those in neonatal rats.21

**Discussion**

These studies are the first to examine the membrane currents of single cardiomyocytes loaded with iron, either in vivo or in vitro. Iron overload, both in vivo and in vitro, had similar effects on cellular iron accumulation and on
Na$^+$ and K$^+$ currents of single cardiomyocytes from 2 closely related rodent species. The gerbils treated with repeated injections of iron dextran over a period of months developed a cardiomyopathy that mimics the cardiomyopathy that develops over a period of years in patients with chronic iron overload.$^5,9$ Because knowledge of cardiac electrophysiology in the gerbil is limited, we also studied the rat, a species in which knowledge of cardiac electrophysiology is extensive.$^{25,26}$ Because the rat can excrete excess iron, cardiac iron deposition could not be produced in vivo in this species. Instead, isolated rat cardiomyocytes were exposed to iron in vitro. Given the 2 different methods of iron loading, the correlation of the changes in Na$^+$ and K$^+$ currents between the 2 models suggests that acutely loaded rat cardiomyocytes may be useful surrogates for determining the specific ion channel targets of iron cardiotoxicity. This is especially important for K$^+$ currents because a number of molecular candidates for I$_{to}$ have been described in rats$^{25}$ and humans.$^{26}$

The mechanism whereby iron reduces the number of functional cardiac Na$^+$ channels and enhances the inactivated state of those channels that remain functional is unknown. Iron may produce peroxidative damage to DNA$^{27}$ and to membrane lipids and proteins.$^{28,29}$ Our antibody experiments suggest that channel production is unaffected. The enhancement of the inactivated state is also consistent with direct modification of Na$^+$ channels, although involvement of the

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**Figure 6.** Sodium-channel protein in control and iron-treated rat cardiomyocytes. A, Western blot of Na$^+$ channel protein in controls and after iron-loading. B, Average values of densitometry on blots from 4 control and 4 test Western blots.

**Figure 7.** K$^+$ currents in gerbil cardiomyocytes. A, Whole-cell K$^+$ currents in cardiomyocytes isolated from control and iron-loaded (8 weeks) gerbils. Currents were elicited with test potentials from $-130$ mV to $50$ mV in 10-mV steps; holding potential was $-100$ mV. B, Average steady-state (filled symbols) and peak currents (open symbols) in control (squares; n=6) and iron-loaded (circles; n=6) cardiomyocytes. *P*<0.05. C, Steady-state inactivation of I$_{to}$ in control (squares; $V_0$, $-57.7\pm0.5$ mV; k=$5.1\pm0.4$ mV; n=5) and iron-loaded (circles; $V_0$, $-52.3\pm1.8$ mV; k=$5.2\pm0.4$ mV; n=5) cardiomyocytes (*P*<0.05). I$_{to}$ was measured as difference between Ipeak and I$_{to}$. D, Recovery of I$_{to}$ from inactivation at $-100$ mV in control (squares; $r=1396\pm53$ ms; n=5) and iron-loaded (circles; $r=1428\pm73$ ms; n=6) cardiomyocytes.
membrane lipid in which the channels are embedded cannot be excluded.\textsuperscript{30} In cultured rat cardiomyocytes, in vitro treatment with iron altered membrane fatty acids\textsuperscript{31} and suppressed mitochondrial respiratory enzymes, with a concomitant reduction in cellular ATP.\textsuperscript{32} The decrease in ATP might alter phosphorylation of Na\textsuperscript{+} channel protein, which affects steady-state inactivation and recovery from inactivation.\textsuperscript{33,34} Whatever the underlying mechanism, the loss of functional Na\textsuperscript{+} channels and enhanced Na\textsuperscript{+} channel inactivation cause the reduction in the overshoot of CAP in the iron-loaded cardiomyocytes.

The relatively slow recovery from inactivation of I\textsubscript{K} in gerbil cardiomyocytes resembles neonatal rat cardiomyocytes and differs from human epicardial myocytes. However, I\textsubscript{K} recovery in human subendocardial myocytes is also slow\textsuperscript{35} and, in humans, heart rate is 5 times slower than in gerbils.

We do not know the molecular species responsible for K\textsuperscript{+} currents in gerbil cardiomyocytes and their involvement in the increase in I\textsubscript{K}. In neonatal rats, as well as in humans, possible candidates for I\textsubscript{K} are Kv4.2/4.3, Kv1.4,\textsuperscript{36,37} and a mitochondrial respiratory enzymes, with a concomitant re-
names. The specific K\textsuperscript{+} channel affected by iron may, there-
the mechanism for the increase, although altered K\textsuperscript{+} channel phosphorylation might contribute.\textsuperscript{38} The contrast with the reduction in Na\textsuperscript{+} current makes it clear that iron does not simply produce nonspecific damage to ion-channel proteins in general. Moreover, the effect of iron on K\textsuperscript{+} currents was specific: I\textsubscript{K} and I\textsubscript{K1} were unaffected. The partial depolariza-
tion of iron-loaded gerbil cardiomyocytes is not due to a change in I\textsubscript{K1} and may result from loss of Na/K ATPase activity.\textsuperscript{39}

The reduction in action potential overshoot will compromise propagation of the cardiac impulse, and increase in I\textsubscript{K} may foreshorten CAP. These effects, together with a heterogeneous pattern of iron deposition, may enhance QT dispersion; clinical evidence of this exists in thalassemia major patients with iron overload. In a preliminary study, QT dispersion was measured as the difference in QT interval among 12 leads of the surface ECG and calculated as QT\textsubscript{max}–QT\textsubscript{min}: 14 of 24 patients had increased (>60 ms) QT dispersion (G.M. Brittenham, unpublished data). Reduced propagation and increased QT dispersion may provide a substrate for the cardiac arrhythmias of iron cardiomyopa-thies. The specific K\textsuperscript{+} channel affected by iron may, there-
be, target for treatment of the arrhythmias\textsuperscript{1,2} caused by iron-overload cardiomyopathy.

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