Increased Release of Arachidonic Acid and Eicosanoids in Iron-Overloaded Cardiomyocytes

Rafael Mattera, PhD; Gregory P. Stone, BA; Nael Bahhur, BA; Yuri A. Kuryshchev, PhD

**Background**—Patients with transfusional iron overload may develop a life-limiting cardiomyopathy. The sensitivity of lipid-metabolizing enzymes to peroxidative injury, as well as the reported effects of arachidonic acid (AA) and metabolites on cardiac rhythm, led us to hypothesize that iron-overloaded cardiomyocytes display alterations in the release of AA and prostaglandins.

**Methods and Results**—Neonatal rat ventricular myocytes (NRVMs) cultured for 72 hours in the presence of 80 μg/mL ferric ammonium citrate displayed an increased rate of AA release, both under resting conditions and after stimulation with agonists such as [Sar1]Ang II. Although iron treatment did not affect overall incorporation of [3H]AA into NRVM phospholipids, it caused a 2-fold increase in the distribution of precursor in phosphatidylcholine species, with a proportional decrease in phosphatidylserine, phosphatidylserine, and phosphatidylethanolamine. Increased release of AA in iron-overloaded NRVMs was reduced by the diacylglycerol lipase inhibitor RHC80267 but was largely insensitive to inhibitors of phospholipases A2 and C. Iron-overloaded cardiomyocytes also displayed increased production of eicosanoids and induction of cyclooxygenase-2 after stimulation with interleukin-1α.

**Conclusions**—Iron overload enhances AA release and incorporation of AA into phosphatidylcholine, as well as cyclooxygenase-2 induction and eicosanoid production, in NRVMs. The effects of AA and metabolites on cardiomyocyte rhythm suggest a causal connection between these signals and electromechanical alterations in iron-overload-induced cardiomyopathy. (Circulation. 2001;103:2395-2401.)

**Key Words:** cardiomyopathy ■ prostaglandins ■ signal transduction ■ myocytes ■ iron

Iron-induced myocardial disease is a life-limiting complication in patients with thalassemia major and other transfusion-dependent refractory anemias, as well as in hereditary hemochromatosis.1 Neonatal rat ventricular myocytes (NRVMs) cultured in the presence of iron and Mongolian gerbils subjected to chronic iron overload have been used as models of clinical cardiomyopathy.2,3 Acute iron loading in cultured rat myocardial cells results in peroxidative injury, decreased activity of mitochondrial respiratory enzymes, impaired contractility, and altered rhythmicity; these specific in vitro alterations can be reversed by metal chelation.2,4–6 Although production of hydroxyl radical (•OH) and lipid peroxidation1,7 are important in initiation of iron-overload–dependent cardiotoxicity, limited information is available regarding downstream signaling alterations. Precedents on enhanced activity of lipases and arachidonic acid (AA) release during oxidative stress8–10 and the role of lipid peroxides in cyclooxygenase activation,11 as well as the reported effects of eicosanoids on cardiac rhythm,12 led us to hypothesize that iron-overloaded cardiomyocytes display changes in AA release and/or production of oxidation metabolites that may contribute to the clinical cardiomyopathy.

**Materials**

Trypsin (type I, 9000 U/mg), AA (free acid), ferric ammonium citrate, cytosine β-d-arabinofuranoside (ara-c), Sar-Arg-Val-Tyr-Ile-His-Pro-Phe ([Sar1]Ang II), BSA, and the lactate dehydrogenase (LDH) kit were purchased from Sigma Chemical Co. Collagenase (type 2, 317 U/mg) was from Worthington. Myo-[2-3H]inositol and [5,6,8,9,11,12,14,15-3H]AA (60 to 100 Ci/mmol) were from New England Nuclear. Enzyme immunoassay kits for prostaglandin (PG) E2, PGE2, and 6-keto-PGF1α, polyclonal anti–cyclooxygenase-2 antiserum, and N-[2-(cyclohexyloxy)-4-nitropheno]methanesulfonamide (NS-398) were purchased from Cayman, and DMEM/F12, inositol-free DMEM (high glucose), and gentamicin were from GIBCO-BRL. Sprague-Dawley pregnant-timed rats were purchased from Zivic-Miller (Portersville, Pa). I,6-Bis-(cyclohexyloxiranoxy)-4-nitropheno]methanesulfonamide (NS-398) was from Worthington, and arachidonoyl-trifluoromethyl-ketone (AACOCF3), 1-(3-(17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrrole-2,5-dione (U73122), and E-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (HELSS) were purchased from Calbiochem. Candesartan and PD123319 were obtained from J.G. Douglas (Case Western Reserve University). Human recombinant interleukin-1α (IL-1α) was a gift from R. Chizzonite (Hoffmann-La Roche, Nutley, NJ) and Martha Konieczkowski (Case Western Reserve University, Cleveland, Ohio).

**Methods**

Tris HCl, pH 7.4; 100 mM NaCl; 5 mM CaCl2; 0.2% Triton X-100; 1 mg/mL BSA; 1 mg/mL collagenase; 0.1 mM dithiothreitol; and 100 μM phenylmethylsulfonyl fluoride (PMSF). Glucose (5 mM), L-glutamine (1 mM), L-ascorbic acid (50 μM), gentamicin (200 μg/mL), and amphotericin (50 μg/mL) were also included in the culture medium. Myc vector was a gift from R. Chizzonite (Hoffmann-La Roche, Nutley, NJ) and Martha Konieczkowski (Case Western Reserve University). Human recombinant interleukin-1α (IL-1α) was a gift from R. Chizzonite (Hoffmann-La Roche, Nutley, NJ) and Martha Konieczkowski (Case Western Reserve University, Cleveland, Ohio).
NRVM Preparation and Iron Treatment

Cell isolation was based on a previously reported procedure. The yield of the preparations was ~3 to 4×10^6 cells/heart. After isolation, NRVMs were resuspended at ~4×10^6 cells/mL in DMEM/F12 medium containing 50 µg/mL gentamicin (DMEM/ F12/GENT) and 10% calf serum. Cells were plated at a density of 0.33×10^6 cells/well of a 12-well plate or 4×10^6 cells per 100-mm dish and cultured at 37°C under humidified atmosphere (95:5, air:CO2). After overnight incubation, the serum concentration was reduced to 0.4% by 2 consecutive 5-fold dilutions with serum-free DMEM/F12/GENT. Cells were subsequently cultured for 72 hours with or without 80 µg/mL elemental iron (supplied as 470 µg/mL ferric ammonium citrate, based on 17% iron content in this salt). Medium was partially renewed every 24 hours (removal of half volume, followed by addition of an equal volume of fresh DMEM/ F12/GENT with or without iron). This resulted in serum concentrations ranging from 0.4% to 0.1% throughout the 72-hour culture. Cultured NRVMs displayed spontaneous contractility after 2 days in culture. Iron treatment decreased cardiomyocyte size, thinned its projections, reduced the number of spontaneously beating cells, and resulted in siderosome accumulation. LDH activity in extracellular medium of control and iron-treated cells (72-hour treatment) was 14±3% and 42±2%, respectively, of total activity in Triton X-100 cell lysates (means±SD, n=4). Control and iron-treated NRVM cultures exhibited 13±3% and 13±1% Trypan blue–positive cells, respectively (mean±SD, n=4); the number of cells in the iron-treated cultures relative to the controls was 0.8±0.2 (mean±SD, n=4).

AA Release

Assays were adapted from a previous study. Briefly, NRVMs cultured in 12-well plates were labeled for 15 to 18 hours in 1.2 mL/well of DMEM/F12/GENT containing 0.7 µCi of [3H]AA and 0.1% BSA (≈90% of precursor was incorporated). Labeled cells were washed at 37°C with DMEM/F12/GENT plus 0.1% BSA. Assays were started by removal of wash media and addition of 1 mL of DMEM/F12/GENT supplemented with 0.1% BSA, 100 µmol/L unlabeled AA (unless otherwise indicated), and agonists and/or enzyme inhibitors. After incubation at 37°C for the indicated times, samples of media were collected, centrifuged at 14 000g for 2 minutes, and counted. [3H]AA release was normalized by measuring [1H] dpm in cell lysates (1 mol/L HONa) and expressed as a percentage of incorporated [3H] dpm.

Distribution of [3H]AA Into Individual Phospholipid Species

Control and iron-treated NRVMs were labeled with [3H]AA for 1 to 24 hours. Labeled cells were washed and extracted with chloroform-methanol-water (final ratio 2:1:0.8 by volume). Lower phases were dried under nitrogen, dissolved in chloroform/methanol/water (75:25:3 by volume), and applied onto silica gel plates previously dried under nitrogen, dissolved in chloroform:methanol:water (final ratio 2:1:0.8 by volume). Lower phases were collected and centrifuged for 15 minutes at 14000g and room temperature. Concentrations of PGE2, PGF2α, and 6-keto-PGF1α (stable metabolite of PGI2) in supernatants were determined by enzyme immunoassay.

Results

Alterations in AA Release

We studied NRVMs treated with ferric ammonium citrate and low serum concentrations (≈0.4% to 0.1% throughout the 72-hour culture). This presentation of iron to cardiomyocytes mimics the situation in patients with transfusional iron burden, who not only display transferrin saturation but also high levels of chelatable, low-molecular-weight, non-transferrin-bound plasminic iron.7 Transferrin-independent iron uptake involves participation of ferrireductases and low-affinity/high-capacity mechanisms.15 Unlike transferrin-dependent uptake, which desensitizes at high concentrations of iron, non-transferrin-bound iron uptake becomes 300-fold higher than the former in cultured cells. Treatment of NRVMs with ferric ammonium citrate results in intracellular accumulation comparable to levels in patients with significant iron burden.16 NRVMs treated with this ferric complex constitute a model of the iron-overloaded heart, given the observed metabolic alterations, ultrastructural pathology, and changes in the overshoot of the action potential and rhythm.2,4–6,13

We studied the effect of iron overload on AA release in resting NRVMs or after activation of angiotensin II (Ang II) receptors, a pathway relevant to cardiac pathophysiology. NRVMs cultured for 72 hours in the presence or absence of ferric ammonium citrate and subjected to overnight labeling with [3H]AA displayed a significant increase in AA release, both under resting conditions and after stimulation with [Sar1]Ang II (a nonselective Ang II type 1 [AT1]/Ang II type 2 [AT2] receptor agonist with reduced susceptibility to amineopeptidases; Table). Similar increases in AA release in iron-overloaded NRVMs were observed after stimulation with thrombin or lysophosphatidic acid (not shown). We routinely add 100 µmol/L unlabeled AA to the assay medium, to minimize reuptake of released [3H]AA. However, increased [3H]AA release in iron-overloaded NRVMs was not linked to this assay condition, because significant effects were also measured with medium containing 0 to 10 µmol/L unlabeled AA (Table). The effect of iron on [3H]AA release was concentration dependent: whereas no changes were detected in NRVMs cultured for 72 hours with 5 µg/mL iron, significant increases were observed after treatment with concentrations ≥20 µg/mL (not shown). All subsequent studies were performed with NRVMs cultured for 72 hours with or without 80 µg/mL iron. Time courses of [3H]AA release showed significantly higher rates in iron-treated cells either in presence or absence of agonist (Figure 1). As shown in Figure 2, increased response to [Sar1]Ang II in iron-overloaded NRVMs was characterized by a higher efficacy and unchanged potency compared with control cells (EC50 0.2±0.1 nmol/L and 0.16±0.03 nmol/L for control and

Eicosanoid Production

NRVMs cultured for 72 hours in the presence or absence of 80 µg/mL iron were treated with or without 10 ng/mL IL-1α during the last 12 to 18 hours of culture. After treatment, extracellular medium was collected and centrifuged for 15 minutes at 1400g and room temperature. Concentrations of PGE2, PGF2α, and 6-keto-PGF1α (stable metabolite of PGI2) in supernatants were determined by enzyme immunoassay.
Increased Release of [3H]AA in Iron-Treated NRVMs

<table>
<thead>
<tr>
<th>AA [μmol/L]</th>
<th>Control</th>
<th>Iron-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>0</td>
<td>0.40±0.02</td>
</tr>
<tr>
<td>[Sar]1Ang II</td>
<td>0</td>
<td>0.51±0.01</td>
</tr>
<tr>
<td>Resting</td>
<td>10</td>
<td>1.10±0.004</td>
</tr>
<tr>
<td>[Sar]1Ang II</td>
<td>10</td>
<td>1.92±0.08</td>
</tr>
<tr>
<td>Resting</td>
<td>100</td>
<td>1.82±0.12</td>
</tr>
<tr>
<td>[Sar]1Ang II</td>
<td>100</td>
<td>3.72±0.12</td>
</tr>
</tbody>
</table>

[Sar]1Ang II-labeled NRVMs were washed and assayed in 30-minute incubations, with or without 0.5 μmol/L [Sar]1Ang II, in the presence of the indicated concentrations of unlabeled AA. Values are means±SEM (n=3) and represent 3 independent experiments.

*P<0.05 and †P<0.01 vs controls (Student’s 2-tailed t test).

Iron treatment of cells, respectively (average and range, n=2). The AA release driven by [Sar]1Ang II in NRVMs exhibited a pharmacology consistent with participation of AT1 receptors (agonized by candesartan, but insensitive to PD123319; Figure 3). The iron-overload–dependent increase in AA release was observed either in NRVMs cultured under standard conditions (low serum, maintaining nonmyocyte cardiac release was observed either in NRVMs cultured under standard conditions (low serum, maintaining nonmyocyte cardiac release was observed either in NRVMs cultured under standard conditions (low serum, maintaining nonmyocyte cardiac

**Figure 2.** Concentration-response relationship for effect of [Sar]1Ang II on [3H]AA release in control and iron-treated NRVMs. Cells were labeled with [3H]AA and incubated for 40 minutes at 37°C. Results are means±SEM of triplicate determinations and represent 2 independent experiments with different preparations of NRVMs.

**Figure 3.** Inhibition of [Sar]1Ang II–dependent release of [3H]AA in NRVMs by AT1 receptor– but not AT2 receptor–selective antagonists. Labeled cells were washed and preincubated for 10 minutes at 37°C in presence or absence of antagonists, followed by incubation with or without [Sar]1Ang II (10 μmol/L) for 40 minutes at 37°C (agonists were also present during incubation). Results are means±SEM of triplicate determinations and represent 2 independent experiments, with different preparations of NRVMs. **P<0.01, significantly different effects of [Sar]1Ang II compared with corresponding responses without antagonists (Student’s 2-tailed t test). Ctrl indicates control; incor., incorporation.
ever, increased labeling of PC in iron-overloaded NRVMs results in this being the predominantly labeled phospholipid class, as opposed to higher labeling of PE in controls (Figure 6).

**Prostanoid Production**

We also studied whether iron overload triggered changes in production of PGE$_2$, PGF$_{2alpha}$, and PGI$_2$, the major eicosanoids secreted by cultured cardiomyocytes. Cells were stimulated with IL-1alpha, which markedly increases prostanoid production in NRVMs compared with agonists such as Ang II or endothelins (not shown). Iron-overloaded cells stimulated with IL-1alpha displayed increased production of PGE$_2$, PGF$_{2alpha}$, and 6-keto-PGF$_{1alpha}$ (stable metabolite of PGI$_2$; Figure 7) compared with controls. The increase in IL-1alpha-induced PGE$_2$ production driven by iron overload was sensitive to 30 \(\mu\)mol/L indomethacin, consistent with this effect representing cyclooxygenase (COX) activity; however, a fraction of the PGE$_2$ production in cytokine-stimulated control cells was insensitive to this treatment and may reflect isoprostanes (not shown).

The higher resting levels of prostaglandin production in iron-overloaded cells seemed to originate from increased COX activity, as measured after 15-minute incubation with 50 \(\mu\)mol/L unlabeled AA (production of PGE$_2$ was 1.1 \(\pm\) 0.1 and 2.1 \(\pm\) 0.1 ng/mg in control and iron-treated NRVMs, respectively; means \(\pm\) SEM; \(n=4\)). Iron treatment also enhanced IL-1alpha-dependent COX-2 induction in NRVMs (bottom panel of Figure 7), whereas increased production of PGE$_2$ in cytokine-stimulated cells (either control or iron-overloaded) was blocked by the COX-2-selective inhibitor NS-398 (not shown).

**Discussion**

Ubiquitous iron-regulated RNA-binding proteins sense intracellular chelatable pools of iron and control expression of target genes by regulating either translation or stability of mRNAs containing 1 or more iron-responsive elements in their 5’ or 3’ regions, respectively.\(^{20}\) Genes regulated by this mechanism include H- and L-ferritin, erythrocyte 5-aminolevulinic synthase, mitochondrial aconitase, transferrin receptor, and Nramp2/DCT1 intestinal iron transporter.\(^{20}\) Although significant advances were made in understanding regulation of genes affecting iron homeostasis, limited information is available regarding signals altered by iron overload. Exceptions to this situation are the effects of iron on expression of protein kinase C-\(\beta\)\(^{21}\) and fibrogenic genes.\(^{22}\) This limitation hinders the treatment of transfusional iron-dependent cardiomyopathy, a life-limiting complication in inadequately chelated patients.\(^{1}\)

We showed that iron overload increases AA release and PC labeling, as well as COX activity, COX-2 induction, and eicosanoid production, in NRVMs. Release of AA is catalyzed by PLA$_2$ enzymes and/or DAG lipase. The PLA$_2$ superfamily includes at least 10 different groups displaying differences in primary structure, cellular localization, and
Ca\textsuperscript{2+} sensitivity. Most of the PLA\textsubscript{2} activity in cardiomyocytes is Ca\textsuperscript{2+}-independent. The results obtained with the inhibitors AACOCF\textsubscript{3} (selective for both group IV PLA\textsubscript{2} and group VI-Ca\textsuperscript{2+}-independent PLA\textsubscript{2}), HELSS (selective for group VI-Ca\textsuperscript{2+}-independent PLA\textsubscript{2}), and RHC80267 (DAG lipase-selective) are consistent with an at least partial participation of the latter in increased AA release in iron-overloaded cells. However, 2 forms of Ca\textsuperscript{2+}-independent PLA\textsubscript{2} (82- and 40-kDa, respectively), exhibiting differences in intracellular localization and HELSS sensitivity, were evidenced in cardiomyocytes. The possibility that iron overload may affect a HELSS-insensitive Ca\textsuperscript{2+}-independent PLA\textsubscript{2} deserves further exploration.

Interestingly, the distribution of labeled \[^{3}H\]AA incorporated in phospholipids of iron-overloaded NRVMs shows an increase in PC (Figure 6). Incorporation of \[^{3}H\]AA may predominantly reflect phospholipid remodeling through the deacylation-acylation Lands cycle, as opposed to de novo glycerophospholipid biosynthesis through the Kennedy cycle, whereas time-dependent remodeling of \[^{3}H\]AA from PC into PE may represent coenzyme A–independent transacylase activity. Iron treatment of NRVMs may increase availability of lysophospholipid acceptors for formation of PC and/or decrease AA remodeling into PE.

The association of changes in AA release and activity of AA-releasing enzymes with myocardial alterations is not unprecedented. Levels of unsterified fatty acids, particularly AA, increase in ischemic myocardium, preceding losses in total phospholipids. Similarly, translocation of Ca\textsuperscript{2+}-independent PLA\textsubscript{2} from cytosol to membranes and protection by its selective inhibitor HELSS were reported in ischemic hearts.

The observation that iron overload sensitizes NRVMs to COX-2 induction (Figure 7) is also significant. Altered eicosanoid production in NRVMs may be responsible for either phenotypic protection or damage during iron overload. Cotreatment with 50 to 500 nmol/L PGF\textsubscript{2\alpha} prevented morphological changes and the decreased beating in iron-overloaded NRVMs, whereas opposite effects were observed with the PGI\textsubscript{1} analog beraprost (R.M. and Y.A.K., unpublished data, 2000). The observations with PGF\textsubscript{2\alpha} are consistent with its hypertrophic effects in cardiomyocytes and with the compensatory role of COX-dependent signals during oxidative stress.
The reported effects of AA metabolites on myocardial rhythmicity suggest a causal connection between our observations and iron-overload–induced electromechanical changes. Indeed, PGE₂, PGI₂, PGF₂, and thromboxane A₂–mimetic compounds trigger reversible tachyarrhythmias, characterized by increased beating rate and contractures. Conversely, prostacyclin (PGI₁) reduces contraction rate in cardiomyocytes. Interestingly, iron overload not only potentiates IL-1α–induced eicosanoid release but also modified the relative ratio of eicosanoid production in cytokine-stimulated NRVMs. Although PGI₁ was the most abundant product in resting NRVMs (controls or iron-overloaded), a relatively higher increase in PGE₂ was observed after IL-1α treatment of iron-overloaded cells compared with smaller changes in either PGI₁ or the less abundant PGF₂ (30-fold versus 3- to 4-fold, respectively, in Figure 7). Consequently, PGE₂ was the predominant eicosanoid in cytokine-stimulated iron-overloaded NRVMs. This is significant because the specific pattern of eicosanoid production in iron-overloaded cardiomyocytes may be ultimately responsible for the final balance of proarrhythogenic or antiarrhythmogenic effects. Reasonable targets for a role of altered production/metabolism of AA in the electromechanical derangement in iron-overload hearts include Na⁺ and K⁺ currents. Precedents supporting this possibility are the reduced Na⁺ currents and increased transient outward K⁺ current (Iₒ) in iron-overloaded gerbil cardiomyocytes, together with the inhibition of cardiac Na⁺ currents by acute treatment with exogenous AA and other polyunsaturated fatty acids. In support of this hypothesis, we observed that chronic treatment of NRVMs with AA (72 hours, 10 μmol/L) decreased Na⁺ currents and increased Iₒ density (Y.A.K. and R.M., unpublished data, 2000). Interestingly, iron-overloaded gerbil hearts exhibit decreased conduction velocity (K. Laurita, PhD, unpublished data, 2000), consistent with reduced Na⁺ currents in isolated cardiomyocytes. Whereas reduced cardiac Na⁺ currents may underlie antiarrhythmic effects of n-3 polyunsaturated fatty acids during delayed afterdepolarizations and triggered activity, a slowing in conduction velocity and the possible shortening of action potentials and refractoriness (due to increased Iₒ), combined with increased size of iron-overloaded hearts, may allow reentry arrhythmias in this cardiomyopathy. The possible consequences of increased release of AA in Ang II–stimulated iron-overloaded NRVMs on cardiac phenotype deserve consideration. This peptide regulates contraction and growth of cardiomyocytes, as well as hyperplasia and matrix production in cardiac fibroblasts. Activation of AT₁ receptors is relevant to mechanical strain–dependent hypertrophy (through recruitment of mitogen-activated protein kinases) and stretch-mediated apoptosis in myocytes. Given the effects of AA on activation of mitogen-activated protein kinases and apoptosis, it is possible to speculate that increased Ang II–dependent AA release may contribute to progression from hypertrophy to heart failure in iron-overload–induced cardiomyopathy. The interaction between cardiac nonmyocyte cells and cardiomyocytes and the relative expression of Ang II receptors in these cell types received attention. The identification of different mechanisms responsible for activation of extracellular signal–regulated kinases by Ang II in rat neonatal cardiac fibroblasts and myocytes supports expression of receptors in both cell types. Using NRVMs cultured with ara-c (to minimize fibroblast proliferation), we observed similar iron overload–dependent increases in AA release and also increased production of PGE₂ and PGF₂ (albeit to a lower extent than in standard cultures; not shown), consistent with a significant role of cardiomyocytes in these responses. However, interactions between cardiomyocytes and nonmyocytes in the alterations driven by iron overload cannot be excluded. Additional studies are required to determine whether the present observations extend to the animal model of cardiac overload and their incidence in human cardiomyopathy. Although the connection between altered production/metabolism of AA and electromechanical alterations in this cardiomyopathy requires additional investigation, the present observations represent an advance in the identification of pathways that either cause or represent adaptive responses to cardiac iron overload.

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