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# Increased expression of the $F_1F_o$ ATP synthase in response to iron in heart mitochondria

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**The objective of the present study was to identify mitochondrial components associated with the damage caused by iron to the rat heart. Decreased cell viability was assessed by increased presence of lactate dehydrogenase (LDH) in serum. To assess the functional integrity of mitochondria, Reactive Oxygen Species (ROS), the Respiratory Control Ratio (RCR), ATP and chelatable iron content were measured in the heart. Chelatable iron increased 15-fold in the mitochondria and ROS increased by 59%. Deterioration of mitochondrial function in the presence of iron was demonstrated by low RCR (46% decrease) and low ATP content (96% decrease). Using two dimensional gel electrophoresis (2DE), we identified alterations in 21 mitochondrial proteins triggered by iron overload. Significantly, expression of the α, β, and d subunits of $F_1F_o$ ATP synthase increased along with the loss of ATP. This suggests that the $F_1F_o$ ATP synthase participates in iron metabolism. [BMB reports 2008; 41(2): 153-157]**

## INTRODUCTION

The heart is a pumping organ that performs mechanical work using ATP. Cardiac energy is generated by oxidative phosphorylation in mitochondria, and mitochondria are rich in respiratory enzymes and related oxidative enzymes. Cellular iron, an essential component of these enzymes, is mainly transported into mitochondria and utilized for the synthesis of heme and iron-sulfur clusters (1). However, excess iron is harmful because the Fenton reaction generates reactive oxygen species (ROS). Free iron has a causative role in ROS-triggered pathological phenomena (2, 3). The protective effect of deferoxamine in myocardial injury supports the hypothesis of iron-induced heart failure (4).

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While excess iron has deleterious effects, few systematic studies have been performed to determine the relevant component(s). On the assumption that the components involved in iron transport and homeostasis may be altered by excess iron, we attempted to identify candidate proteins. In this report, mitochondrial components altered by excess iron are identified using the proteomic approach.

## RESULTS

### Iron content and myocyte viability

As labile iron, which is treated as free iron, exerts toxic effects by producing ROS, Calcein AM was used to measure chelatable labile iron content. The labile iron level of mitochondria increased with number of iron injections from 4-fold in the Iron(1) to 15-fold in the Iron(3) group (Table 1).

The effect of iron overload on heart cell viability was assessed from the level of LDH in sera. The LDH content increased from 2-fold in the Iron(1) group to 3-fold in the Iron(3) group (Table 1). To confirm the toxicity of the iron, isolated cardiac myocytes were incubated in the presence or absence of iron, and their viability compared. Damage due to iron was apparent on the first day, because only 80% as many cells were recovered from the iron overloaded heart as from control heart (Fig. 1A), and few cells survived by day 6, suggesting a long-term effect of the iron. Initial cell attachment, as well as viability seems to be affected. The dependence of cell viability on iron concentration along with the protective effect of deferoxamine (DFO), a specific iron chelator, demonstrates the specificity of the effect of iron (Fig. 1B). The increased LDH in serum and the low viability of cultured primary cardiac myocytes in the presence of iron provide clear evidence of the toxic effect of the metal.

### Heart weight and mitochondrial ATP content, ROS, and RCR of iron-overloaded rat

Iron-overloaded hearts were enlarged, with a 20% increase in weight. This hypertrophy is a typical sign of heart impairment. To determine the effect of excess iron on mitochondria, standard indices of mitochondrial function, such as ATP content, ROS, and RCR, were measured (Table 2). There was consid-

**Table 1.** LDH activity of serum and chelatable iron content of mitochondria

| Group | LDH (units/ml) | Iron*** ( nmol/mg) |
|---|---|---|
| Control | 200.9 ± 31.2 | 0.026 ± 0.007 |
| Iron(1) | 414.5 ± 55.0* | 0.095 ± 0.022 |
| Iron(2) | 517.7 ± 75.7* | 0.290 ± 0.030 |
| Iron(3) | 597.5 ± 114.4** | 0.402 ± 0.063 |

*P < 0.0005, **P < 0.001, ***P < 0.00001

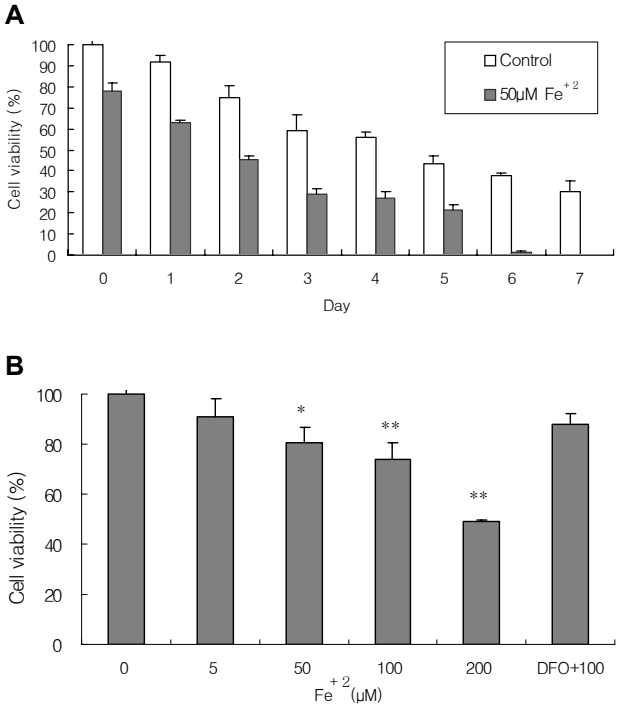


**Fig. 1.** Effects of iron on primary cardiac myocyte viability. Primary cardiac myocytes were isolated from heart ventricles, and incubated for 4 h to attach. The attached cells were transferred to new culture plates supplied with fresh medium, and incubated in the presence of 50 μM $FeSO_4$. (A) After 2 h, cells were washed and incubated for 7 days in the absence of $FeSO_4$. In (B), cardiac myocytes were incubated for 2 h in the presence of various concentrations of $Fe^{2+}$, washed, and counted after 24 h using Trypan Blue. For comparison, either $Fe^{2+}$ was omitted or 1 mM DFO was added to the appropriate cultures. * P < 0.0005, ** P < 0.001.

erable ATP loss in the iron-overloaded mitochondria (less than 5% compared to controls). ROS increased by 56% and RCR decreased by 46%. Lipid peroxidation, a typical damaging effect of iron, should elicit changes in membrane structure and function, and oxidative phosphorylation in the presence of damaged membranes may lead to ATP loss and low RCR (5). Either low state 3 or high state 4 respiration is observed in iron

**Table 2.** Effects of iron overload on heart weight, mitochondrial ATP content, ROS, and RCR

| | Heart wt (g)* | ATP** | ROS | RCR |
|---|---|---|---|---|
| Control | 0.99 ± 0.04 | 27.3 ± 1.3 | 0.34 ± 0.00 | 6.48 ± 0.99 |
| Iron (3) | 1.20 ± 0.13 | 1.0 ± 0.2 | 0.53 ± 0.10 | 3.47 ± 0.36 |

*P < 0.01, **P < 0.05. ATP content in μmol/mg; ROS in arbitrary units at 504 nm.

overload together with the low RCR. State 4 respiration, in particular, is a common consequence of uncoupling (6). Uncoupling often occurs in the presence of iron and consumes ATP (7). The present data obtained with iron overload are in good agreement with the estimates expected for uncoupling. Hence, iron appears to exert its effect by uncoupling. The severe loss of ATP often observed in ischemic hearts (8) supports the argument that iron is a common cause of heart failure.

### Increased α, β, and d subunits of $F_1F_o$ ATP synthase

We investigated the mitochondrial components altered following iron overload using 2D gel electrophoresis. Spots displaying more than 3.5-fold differences (either increases or decreases) were selected for identification (Supplementary Fig. 1). Although the limited information available on rat heart mitochondria did not allow us to identify all the altered spots, the changes identified occurred mostly in the components of oxidative metabolism, including the $F_1F_o$ ATP synthase, electron transport chain, TCA cycle, β-oxidation, and amino acid oxidation, in agreement with earlier reports (Supplementary Table) (9, 10). Interestingly, $F_1F_o$ ATP synthase subunit β and methylmalonate semialdehyde dehydrogenase were significantly elevated. As ATP loss can result from the ATPase activity of $F_1$ components, we focused on $F_1F_o$ ATP synthase, and assessed the accumulation of the $F_1$ α, β, and d subunits by immunoblot analysis (Fig. 2). For β, three spots were identified, one with a normal pI of 5.2 and two with acidic pIs of 4.8 and 5.0, whose expression was substantially increased (6- and 4-fold, respectively) (Supplementary Fig. 2). Conceivably, protein modification, such as phosphorylation, occurs in the presence of iron, although its functional significance is not known. Similarly, in addition to the intact α chain, a C-terminal fragment was identified. A comparable, but not identical, oxidized product was reported by Belogrudov (11). However, the dramatic increase in expression of the β subunits with altered pIs, along with the high levels of intact α and d subunit, point to more than a simple repair process.

## DISCUSSION

It is well known that the toxic effect of iron results from ROS production. The ROS content of the iron-overloaded mitochondria was considerably elevated, as expected. However, the ex-

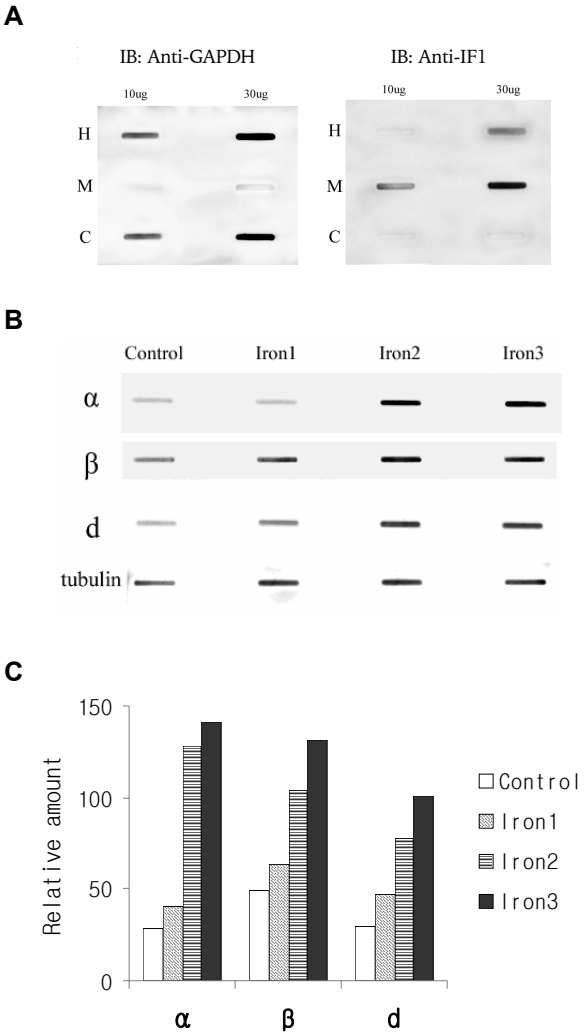


**Fig. 2.** Immunoblot for the α, β, and d subunits of $F_1F_o$ ATP synthase in iron overloaded heart mitochondria. (A) To confirm purity of mitochondrial preparation, proteins from total homogenate (H), mitochondria (M), and cytosolic fraction (C) were subjected to immunoblot analysis using antibodies that recognize glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a cytosolic marker, and ATPase inhibitor protein (IF1) as mitochondrial marker. (B) Sonicated mitochondrial extracts (20 μg) were prepared from rat hearts injected with $FeCl_3$ (0.049 mg/g body weight) once (Iron1 group), three times (Iron2 group), and six times (Iron3 group). The extracts were subsequently incubated with antibodies against α, β, and d subunits of $F_1F_o$ ATP synthase (Mitoscience, USA). Tubulin (Santa Cruz, USA) was employed as the standard. (C) Intensity of immunoblot band in (B) was standardized by that of tubulin.

tremely low level of ATP observed in this study seems significant, as ATP loss is considered a primary cause of cell death in heart failure (12). In an attempt to identify the responsible mitochondrial factor(s), we used a proteomic approach. Expression of the $F_1$ subunits of $F_1F_o$ ATP synthase was found to increase substantially with augmentation of the normal β subunit of pI 5.2, and the appearance of acidic subunits (pI 4.8 and 5.0) along with elevated α and d subunits. As $F_1$ has ATPase activity, ATP depletion appears to be related to the increased expression of these $F_1$ subunits. The apoptosis induced by oligomycin in erythroleukemia cells points to a critical role of $F_1F_o$ ATP synthase in determining cell fate by reducing ATP levels (13). Similarly, overexpression of the α and β subunits by excess iron may stimulate ATPase activity and diminish ATP levels, with fatal consequences.

Belogrudov (11) reported iron-induced oxidative cleavage of the $F_1$ factor, generating a number of α and β fragments. We found a similar, but not identical, C-terminal fragment of the α subunit (residues 376 to 533) in the iron-overloaded heart mitochondria. Fe-catalyzed oxidation and cleavage also occur in the ATPase located in the sarcoplasmic reticulum as a result of binding of the $Fe^{2+}$-ATP complex to the substrate site (14). Increased expression of the α subunit may occur to compensate for the damage resulting from the incomplete fragments. Alternatively, the C-terminal fragment may arise as one of the various splicing products of the α transcript that were recently identified using a cDNA clone (15). Transcriptional/translational regulation via alternative splicing may be altered to maximize cellular activities and survival. Meanwhile no β fragment was detected in this study. However, the additional β spots with acidic pIs (4.8 and 5.0) were suggestive of modification(s), such as phosphorylation/nitrosylation. In fact, phosphorylation on ser/thr residues has been detected by Western analysis (unpublished observation). Recently, phosphorylation of the β subunit was demonstrated in the skeletal muscle of a type 2 diabetes patient together with a possible alteration of ATP synthesis (16), though the physiological significance of the modification is not known.

What benefits does the cell obtain from overproducing $F_1$ α and β subunits in the iron overloaded heart mitochondria? The $F_1$ component or the $F_1F_o$ ATP synthase may participate in iron transport. A similar increase of ATP synthase α and β chains was noted in T. ferrooxidans growing with $Fe^{2+}$ as energy source (17). The dependence of the increase on iron availability may reflect the emergence of a primitive enzyme performing the oxidation/reduction of $Fe^{2+}$ and evolving to carry out oxidative phosphorylation. The original high affinity of the enzyme for iron has been maintained in iron metabolism. The $F_1F_o$ ATP synthase may be involved in iron transport by providing a direct path. Iron transport is known to be energy-dependent (18) and eventually reduces the membrane potential, which needs to be maintained to support cellular activities. One way to maintain the potential is by the operating oxidative phosphorylation in reverse: this ATPase activity of the $F_1F_o$ ATP synthase would restore the membrane potential with consumption of ATP.

If newly synthesized $F_1$ subunits are not properly located or assembled with $F_o$ they may accumulate in the matrix in soluble form. $F_1$ subunits, in the absence of $F_o$, are incapable of ATP synthesis but retain ATPase activity so that ATP may well be consumed by the increased level of α and β subunits. Indeed,

altered assembly and the presence of subcomplexes of the $F_1F_o$ ATP synthase with abnormally high levels of the the $F_1$ component were observed in transformed cell lines containing a subunit 6 mutation (19). Loss of ATP resulting from overproduction of the α and β subunits is consistent with the uncoupling effect of iron. This uncoupling may be beneficial to the cell by lowering membrane potential (20) and reducing the production of ROS by the iron catalyzed Fenton reaction (21, 22). Probably cell survival is better when ATP is reduced than when ROS increase. Otherwise, the high membrane potential would accelerate ROS production in the presence of excess iron.

Proteomic analysis is one of the most powerful methods of identifying cellular components. The study of iron metabolism by this approach may reveal hidden regulatory roles of iron in oxidative phosphorylation. Improvements in solubilization methods for membrane enzymes should enable us to elucidate the mechanism underlying the pathology of heart failure due to iron overload.

## MATERIALS AND METHODS

### Iron injection and isolation of primary cardiac myocytes

Male Sprague-Dawley rats (200-250 g) purchased from Orient (Seoul, Korea) were maintained under standard conditions, with free access to food and water. Animals to be subjected to iron overload were injected hypodermically with $FeCl_3$ (0.049 mg/g body weight) every other day for 2 weeks. The iron-administered rats were divided into three groups, specifically, Iron(1), Iron(2) and Iron(3), receiving one, three and six injections, respectively. Matched control rats were injected with equal amounts of saline. After the rats were killed their hearts were perfused and cardiac myocytes were isolated (23).

### Measurement of ATP content, ROS, and RCR of mitochondria

Heart mitochondria were isolated by differential centrifugation in slightly modified preparation buffer (0.25 M sucrose, 3 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.05% saponin, pH 7.4). ATP was measured according to the method of Lundin and Thore (24). Mitochondria (100 μg) were mixed with 1% TCA and 10 mM Tris-acetate (pH 7.75) along with components of the ENLINER ATP assay kit (Promega, USA), and incubated for 10 sec. Luminescence was measured at 470 nm with a Berthold LB9509 instrument (Germany). To estimate ROS, 330 μM 2′-7′-dichlorodihydrofluorescein diacetate (DCFDA) (Molecular probe, USA) was immediately added to the mitochondrial sample (250 μg/ml) and absorption measured at 504 nm (25). The functional integrity of mitochondria was assessed by RCR. Specifically, after brief incubation (1 min), 0.5 mM ADP was added, and oxygen consumption was measured using a Clark-type oxygen electrode (Hansatech, UK) at 25°C (26). Mitochondria displaying RCR above 4 were used throughout the experiments.

### Cell viability and LDH assay

For measurement of cell viability, 0.4% (w/v) trypan blue was added to the suspension and mixed thoroughly and the cells in a hemocytometer were counted using an inverted microscope (Olympus, Japan). At least four independent counts were performed. The lactate dehydrogenase (LDH) assay was performed using rat serum (20 μl) according to the procedure of Bauer (27).

### Free iron assay

Calcein AM (Invitrogen, USA) was used to measure chelatable (free) iron content (28). Mitochondrial samples (250 μg) were incubated with 0.125 μM Calcein AM for 5 min at 37°C in PBS (pH 7.4). After washing, fluorescence was monitored using a fluorospectrophotometer with excitation at 488 nm and emission at 517 nm (JASCO, Japan).

### 2D gel electrophoresis (2DE)

Heart mitochondria were isolated in preparation buffer (0.25 M sucrose, 3 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.05% saponin, pH 7.4), and their functional integrity was assessed by estimating RCR. One volume of washed mitochondria was mixed with 2 volumes of lysis buffer containing 8 M urea, 4% CHAPS, 40 mM Tris-Cl (pH 7.4), proteinase inhibitor cocktail, and 1 mM NaF. After sonication and centrifugation at 4°C, supernatant fractions (500 μg) were prepared in re-swelling solution (8 M urea, 10% glycerol, 2% IPG pH 3-11) (340 μl). Isoelectric focusing with Immobiline Dry Strip, pH 3-10 (18 cm, Amersham Pharmacia) (29) and SDS polyacrylamide gel electrophoresis (18 by 20 cm, Bio-Rad Protean II) were conducted according to the method of Klose and Kobalz (30). Silver-stained spots were analyzed by SWISS-PROT with PD-Quest 6.0 (Bio-Rad, USA), and identified using the Ettan MALDI-TOF mass spectrometer (Amersham Biosciences, USA). Peptides were evaporated with a $N_2$ laser at 337 m using a delayed extraction approach, and accelerated with 20 kV injection pulse for time-of-flight analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound (http://129.85.19.192/profound_bin/WebProFound.exe) involving peptide mass fingerprinting, developed at the Rockefeller University, was employed for protein identification (Genomine, Pohang). Spectra were calibrated using trypsin autodigestion peptide ion peak signals (*m/z* 842.510, 2211.1046) as internal standards.

Chemicals were purchased from Sigma, unless otherwise indicated. Data are presented as mean values ± SD of at least 5 different experiments in which assays were performed in duplicate. One-way ANOVA was used for statistical comparison between means, and $P < 0.05$ was considered statistically significant.

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