Mitochondrial Cysteine Desulfurase Iron-Sulfur Cluster S and Aconitase Are Post-transcriptionally Regulated by Dietary Iron in Skeletal Muscle of Rats[1]

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ABSTRACT Cysteine desulfurase IscS is required for cellular iron-sulfur protein maturation in eukaryotes and prokaryotes. In this study, we examined the effect of dietary iron intake on the expression in rat skeletal muscle of IscS in relation to 2 iron-sulfur proteins, cytosolic aconitase (c-aconitase) and mitochondrial aconitase (m-aconitase). Three groups of male weanling Wistar rats were used; 1 group was fed an iron-deficient diet (D), and the other 2 groups were pair-fed (P) or freely fed (C) a control (35 mg Fe/kg diet) diet for 1 or 2 wk. At the end of wk 1 and 2, the mitochondrial IscS protein levels in the skeletal muscle of iron-deficient rats had decreased to 45 and 50% of those of the control and pair-fed rats, respectively, whereas the IscS mRNA levels did not differ among the 3 groups, indicating that iron deficiency affected the expression of IscS protein at the post-transcriptional level. Iron deficiency caused a 55–76% reduction in c-aconitase activity and an ~50% reduction in the c-aconitase protein level. The m-aconitase activity and protein level in iron-deficient rats also declined to 50 and 58–64% of the control levels, respectively. Our results indicate that dietary iron modulates mitochondrial IscS protein and aconitase at the post-transcriptional level, and mitochondrial IscS may be associated with this regulation of aconitase in skeletal muscle. J. Nutr. 135: 2151–2158, 2005.

KEY WORDS: • *aconitase* • *cysteine desulfurase* • *iron deficiency* • *rats* • *skeletal muscle*

Iron-sulfur (Fe-S) proteins are proteins carrying Fe-S cluster(s); these include the iron typically tetrahedral coordinated by the thiolate ligands of cysteine side chains with additional coordination to each iron provided by inorganic sulfides (1). Due to their intrinsic redox properties, Fe-S clusters play a variety of biological roles, including facilitation of the electron transfer processes during oxidative phosphorylation, catalysis of enzymatic reactions in aconitase, maintenance of structural integrity in the DNA repair enzyme endonuclease III, and sensing of intracellular iron and/or oxidant levels via iron regulatory protein 1 (IRP1)[3] (2).

The 2 isozymes of aconitase have a similar Fe-S cluster at their catalytic center, but are encoded by different genes (3). The cytosolic aconitase (c-aconitase) is a bifunctional protein that is identical to IRP1; it binds to the stem-loop structure of the iron-responsive element (IRE) in the untranslated region (UTR) of target transcripts including mitochondrial aconitase (m-aconitase). The interconversion between c-aconitase and the IRE-binding activity of IRP1 is regulated by an Fe-S cluster switch. When cellular iron becomes limiting, the c-aconitase loses its Fe-S cluster to function as IRP1 with high IRE-binding affinity; conversely, when cellular iron is replete, IRP1 assembles into a [4Fe-4S] cluster to exhibit aconitase activity and low IRE-binding affinity (4). Thus, iron modulates IRP1 by influencing the amount of the protein that has IRE-binding activity or c-aconitase without changing the total amount of the protein in the cell (5). High IRP-IRE binding inhibits the translation of transcripts with IRE located in the 5′-UTR; alternatively, it enhances the stability of mRNA with IRE located in the 3′-UTR, and vice versa (6). On the other hand, the primary function of m-aconitase is the conversion of citrate to isocitrate in the tricarboxylic acid cycle. It contains a [4Fe-4S] cluster that serves as the enzymatic binding site for citrate, and loss of the cluster results in enzyme inactivation (7). A conserved IRE was found in the 5′-UTR of m-aconitase mRNA; therefore, it is subject to translational regulation by IRP-IRE binding in response to intracellular iron levels, and iron depletion causes a reduction in its protein content and enzyme activity (8–10).

Recent studies in bacteria and yeast led to the identification of a number of mitochondrial proteins that are implicated in the biogenesis of Fe-S clusters (2,11). The nitrogen fixation S (NifS) gene was first identified as an essential member of the nitrogen fixation system, which is solely responsible for the biosynthesis of the Fe-S cluster of nitrogenase (12,13). IscS, a homolog of NifS, was found later and is involved in a general mechanism for Fe-S cluster biogenesis (14). Many eukaryotic IscS homologues have also been identified, including those in yeast (15), mouse (16), and human cell lines (17). Both NifS and IscS are pyridoxal-phosphate–dependent enzymes exhibiting cysteine desulfurase activity; they produce elemental

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[3] Abbreviations used: C, control; c-aconitase, cytosolic aconitase; D, iron-deficient; HDSC, Hepes-dithiothreitol-sucrose-citrate; IRE, iron-responsive element; IRP, iron regulatory protein; Isc, iron-sulfur cluster; m-aconitase, mitochondrial aconitase; 2-ME, 2-mercaptoethanol; NifS, nitrogen fixation S; P, pair-fed; TIBC, total iron-binding capacity; UIBC, unsaturated iron-binding capacity; UTR, untranslated region.

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sulfur from L-cysteine and serve the physiological function of supplying inorganic sulfur for the assembly of Fe-S clusters (12–14). The reaction involves the generation of a persulfide intermediate on a conserved cysteine residue of NifS protein (18) and transfer to scaffold proteins (19–21). The eukaryotic IscS homologous Nfs1p is found mainly in the mitochondrial matrix (15,16); however, several lines of evidence suggested nuclear and extramitochondrial localization for the protein (17,22) as well as additional functions such as thiouridine modification of tRNAs (23,24).

Skeletal muscle is severely affected by iron deficiency (25). Previous studies demonstrated a significant reduction in the myoglobin, mitochondrial cytochrome, cytochrome oxidase, and Fe-S enzyme content of skeletal muscle of iron-deficient rats (26,27). Furthermore, in contrast to causing a slight increase or no change in c-aconitase activity in the liver (28,29), iron deficiency was shown to result in decreased c-aconitase activity in the muscle (30). Because mitochondria play an essential role in the biosynthesis of cellular Fe-S proteins (11), and mutation of *NFS1* gene in *Saccharomyces cerevisiae* causes decreased activities of both the mitochondrial and cytosolic Fe-S proteins (15,31), we hypothesized that the mitochondrial assembly machinery for Fe-S clusters was impaired by iron deficiency. To test the hypothesis, we studied the effect of dietary iron deficiency on the expression of IscS and the 2 aconitase isozymes in rat skeletal muscle.

MATERIALS AND METHODS

Animals and treatment. The iron-deficient diet was based on a modified AIN-76 formulation (32) in which an AIN-76 mineral mixture with iron omitted was used, and cornstarch and soybean oil replaced sucrose and corn oil, respectively. The chemical components of the diet were obtained from Sigma Chemical or other equivalent suppliers. Iron concentration of the iron-deficient diet was measured to be 2 mg Fe/kg diet by atomic absorption spectrophotometry (Perkin Elmer Model 3100). The control diet (35 mg Fe/kg) was prepared by adding $FeSO_4 \cdot 7H_2O$ (Sigma Chemical) to the iron-deficient diet and the iron concentration was also verified.

Weanling male Wistar rats ($n = 24$; Laboratory Animal Center, College of Medicine, National Taiwan University), initially weighing 55 ± 4 g, were housed individually in stainless steel cages with wire-mesh floors in a temperature- (22 ± 2°C), humidity-, and light-controlled room. Animal care and handling conformed to National Science Council guidelines. Rats were randomly assigned to 1 of 3 treatment groups: the control group (C) was fed the control diet, the iron-deficient group (D) was fed the iron-deficient diet, and the pair-fed group (P) was also fed the control diet. Each pair of iron-deficient and pair-fed rats was matched by body weight. The amount of diet provided to a pair-fed control rat was equal to the consumption of its Fe-deficient partner on the previous day. All of the rats had free access to deionized water. Body weight and feed intake were measured daily. At the end of wk 1 and 2, 4 rats from each group were deprived of food overnight and killed by carbon dioxide asphyxiation between 900 and 1100 h in the morning. Blood were collected for hemoglobin analysis and serum preparation. Serum was separated by centrifugation at 1200 × *g* for 15 min and was frozen at −70°C until analyzed. Both the right and left hind limb muscles (gastrocnemius, plantaris, and soleus muscle) were excised and weighed. The left hind limb muscles were used immediately to prepare cytosolic and mitochondrial fractions. The right hind limb muscles were quick-frozen in liquid nitrogen and stored at −70°C for RNA extraction.

Fractionation of muscle cytosol and mitochondria. The cytosolic and mitochondrial fractions were separated by a modified procedure of fractional extraction described previously (33,34). Briefly, the minced muscle was suspended in cold HDSC buffer (50 mmol/L Hepes, 1 mmol/L dithiothreitol, 0.25 mol/L sucrose, 2 mmol/L citrate, 0.5 mg/L leupeptin, 0.2 mmol/L phenylmethylsulfonyl fluoride, pH 7.4) and stirred magnetically on an ice bath for 30 min. The supernatant was collected after centrifugation at 10,500 × *g* for 60 min and used for analysis of c-aconitase activity and IRE-binding activity. The pellet was further homogenized and centrifuged at 700 × *g* for 10 min. The supernatant was collected and then centrifuged at 1000 × *g* for 15 min to pellet the mitochondria. After being washed with buffer twice, the mitochondria were resuspended in HDSC buffer with 1% Triton for analysis of IscS protein, mitochondrial aconitase activity, and its protein. Recovery and cross-contamination of cytosol and mitochondria were calculated from iron-sufficient rats on the basis of the amount of activity of the marker enzymes for cytosol [lactate dehydrogenase (LDH)] and mitochondria [glutamate dehydrogenase (GDH)] recovered in each fraction compared with the sum of activity in the pellet and all supernatant fractions from the centrifugation. The mitochondrial contamination of cytosol was 5 ± 1%, and the recovery of cytosol was 70 ± 5%. Cytosolic contamination of mitochondria was 1 ± 0.2%, and recovery of mitochondria was 27 ± 5%.

Enzyme assays. Activities of GDH (EC 1.1.4.3) and LDH (EC 1.1.1.27) were assayed as described previously (35,36). The activity of aconitase was determined by the coupled reaction of aconitase (EC 4.2.1.3) and isocitrate dehydrogenase (EC 1.1.1.42). One unit of enzyme activity was defined as the reduction of 1 μmol $NADP^+$/(min · mg protein) (37).

Electrophoretic mobility shift assay of IRE-binding activity. IRE-binding activity of IRP1 and IRP2 was determined by gel retardation analysis using a ^{32}P-labeled IRE-containing RNA probe of the first 73 nucleotides from rat L-ferritin 5′-UTR (kindly provided by Dr. R. S. Eisenstein, University of Wisconsin, Madison, WI) in the absence or presence of 5% 2-mercaptoethanol (2-ME) (38). The detailed procedure was described by Huang and Shaw (39). Free and protein-bound [^{32}P] RNA bands were cut from gels and quantified by scintillation counting.

Muscle RNA isolation and Northern blot analysis. IscS and c-aconitase cDNA were prepared by cloning an RT-PCR product of rat liver RNA amplified using the following oligonucleotides: 5′-TAAAGGAACTAGAGGCTGC-3′ (forward) and 5′-TGGGTCCACTTGATGCTC-3′ (reverse) for IscS (bp: 1–1356, accession number: AF336041) and 5′-AAATAAACCCTGTCTGCCCCGC-3′ (forward) and 5′-CGGAGGTGCTTGGTAATGGTGA-3′ (reverse) for c-aconitase (bp:574–1043, accession number L23874). The β-actin cDNA (accession number 55574) was obtained from Huang and Shaw (39) and human m-aconitase cDNA (accession number U80040) was a kind gift from Dr. H. H. Juang (Memorial University of Chang Gung, Taiwan, ROC) (40). Total RNA was extracted using guanidium thiocyanate:phenol:chloroform extraction method (41). The ^{32}P-labeled cDNA probes of IscS, c-aconitase, and m-aconitase were prepared using a cDNA labeling kit (Amersham Biosciences); 30–40 μg of total RNA was separated on 2% formaldehyde-agarose gel, transferred to nylon membrane (Gene Screen Plus; DuPont), and hybridized with ^{32}P-labeled cDNA probe. All of the results were recorded on X-OMAT AR film (Kodak) after stringent washing. Signals were quantified using a microcomputer image analysis system (MCID; Fuji) and normalized to the expression of β-actin.

Western blot analysis. The mitochondrial fraction containing 9.5 μg of protein was used for all Western blot analyses. For IscS analysis, the mitochondrial proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, incubated with rabbit anti-human IscS polyclonal antibodies (1:500 dilution) in NET solution [0.25% (wt:v) gelatin, 0.15 mol/L NaCl, 5 mmol/L EDTA, 0.05% Tween 20, 50 mmol/L Tris-HCl, pH 8.0] at room temperature for 1.5 h. The blot was washed and then incubated with biotinylated goat anti-rabbit IgG (1:5000 dilution, VECTASTAIN ABC kit, Vector Laboratories) for 1 h. This in turn was detected with streptavidin-horseradish peroxidase and ECL Western blotting detection reagents (Amersham Biosciences). For m-aconitase analysis, the mitochondrial proteins were separated by 8% SDS-PAGE and transferred to polyvinylidene fluoride membrane, then incubated with rabbit anti-bovine heart m-aconitase antibodies (1:2000 dilution, a gift from Dr. R. S. Eisenstein, University of Wisconsin-Madison, Madison, WI); this was followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:20,000 dilution, Tropix, Applied Biosystems), and finally visualized with an enhanced chemiluminescence substrate CDP-star (Tropix). The emitted light was captured on BioMax light film (Kodak) and the immunoblots were

TABLE 1

Body weights, feed intakes, and feed efficiency in rats fed control or iron-deficient diets or pair-fed the control diet for 2 wk[1]

	Final body weight		Body weight gain		Food intake		Feed efficiency	
Diet group	Wk 1	Wk 2	Wk 1	Wk 2	Wk 1	Wk 2	Wk 1	Wk 2
	g/rat		*g/d*				*g gain/g feed*	
Control	95 ± 9^{c}	148 ± 17^{a}	6.1 ± 0.8^{ab}	6.7 ± 1.2^{a}	10.2 ± 1.1^{b}	12.5 ± 1.1^{a}	0.60 ± 0.06^{a}	0.54 ± 0.02^{ab}
Fe-deficient	84 ± 3^{c}	128 ± 10^{b}	4.1 ± 0.4^{c}	5.3 ± 0.5^{bc}	8.1 ± 0.8^{c}	10.4 ± 0.8^{b}	0.51 ± 0.07^{b}	0.51 ± 0.01^{b}
Pair-fed[2]	86 ± 8^{c}	119 ± 8^{b}	4.4 ± 0.8^{c}	5.3 ± 0.4^{bc}	8.3 ± 0.7^{c}	10.0 ± 0.7^{b}	0.53 ± 0.06^{ab}	0.53 ± 0.01^{ab}
	P-values from 2-way ANOVA							
Diet	0.0094		0.0003		<0.0001		0.0427	
Time	<0.0001		0.0160		<0.0001		0.2482	
Diet × Time	0.5490		0.8108		0.8146		0.3477	

[1] Values are means ± SD, $n = 4$. Means for each variable without a common letter differ, $P < 0.05$.
[2] Rats were fed the control diet containing 35 mg iron/kg diet to the level of food intake exhibited by their paired partners in the Fe-deficient group.

quantified using an MCID image analysis system. The α-tubulin was used as a loading control using a mouse anti-α-tubulin monoclonal antibody according to the manufacturer's recommendation (Sigma).

Production of IscS antibody. The rabbit anti-human IscS polyclonal antibody was raised against the synthetic peptide RSRKKHLITTQTEHK, which corresponds to amino acid residues 143–157 of human IscS (17). Antiserum was generated by Cashmere Scientific Company. The specificity of the polyclonal IscS antibody was confirmed using purified 53 kDa rat IscS-His_6 fusion protein (results not shown).

Biochemical analyses. Hemoglobin concentration was determined by the cyan-methemoglobin method using Drabkin's solution (42). Serum iron and unsaturated iron-binding capacity (UIBC) were determined using a serum iron/total iron-binding capacity (TIBC) kit (RANDOX). TIBC was calculated as the sum of serum iron and UIBC and transferrin saturation as a percentage of serum iron concentration to TIBC values. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce) with bovine serum albumin as standard.

Statistical analysis. All values are presented as means ± SD. The main effects of diet, time, and interaction between the 2 factors for each dependent variable were tested using 2-way ANOVA, followed by Tukey's test. Correlations between IscS or IRP activity and aconitase were assessed by the Pearson's correlation test. All of the statistical analyses were carried out on a PC version of SAS System (Version 6.12, SAS Institute). Differences were considered significant at $P < 0.05$.

RESULTS

Growth and hematological indices. Diet and time significantly affected body weight, weight gain, and food intake, although there was no interaction between the 2 factors (**Table 1**). All rats had higher body weight, weight gain, and food intake at wk 2 than at wk 1. The Fe-deficient group had significantly lower body weight, weight gain, and food intake than the control group. Iron deficiency resulted in a 12–14% reduction in body weight, a 21–33% reduction in weight gain, and a 17–21% reduction in food intake. The pair-fed group had growth and food intake reductions similar to those of the Fe-deficient group. Diet alone affected feed efficiency, which was lower in the Fe-deficient group only at wk 1 but not at wk 2.

Hemoglobin, serum iron, TIBC, and transferrin saturation were significantly affected by diet; TIBC was affected by time; hemoglobin, serum iron, and TIBC were affected by a diet × time interaction (**Table 2**). The Fe-deficient group became anemic at wk 1, and had a significantly lower hemoglobin

TABLE 2

Hematological indices of iron status in rats fed control or iron-deficient diets or pair-fed the control diet for 2 wk[1]

	Hemoglobin[2]		Serum iron		TIBC		Transferrin saturation	
Diet group	Wk 1	Wk 2	Wk 1	Wk 2	Wk 1	Wk 2	Wk 1	Wk 2
	g/L		*μmol/L*				*%*	
Control	129 ± 6^{ab}	134 ± 7^{a}	26 ± 14^{ab}	16 ± 1^{bc}	98 ± 6^{b}	106 ± 11^{b}	24 ± 13^{ab}	16 ± 1^{bc}
Fe-deficient	79 ± 3^{c}	58 ± 3^{d}	9 ± 4^{c}	7 ± 3^{c}	103 ± 7^{b}	160 ± 13^{a}	8 ± 3^{cd}	4 ± 2^{d}
Pair-fed	124 ± 5^{b}	130 ± 6^{ab}	19 ± 8^{ab}	30 ± 4^{a}	84 ± 6^{c}	98 ± 17^{b}	27 ± 1^{ab}	32 ± 10^{a}
	P-values from 2-way ANOVA							
Diet	<0.0001		<0.0001		<0.0001		<0.0001	
Time	0.0855		0.8228		<0.0001		0.4995	
Diet × time	<0.0001		0.0312		<0.0001		0.1737	

[1] Values are means ± SD, $n = 4$. Means for each variable without a common letter differ, $P < 0.05$.
[2] Hemoglobin MW = 64,458 Da.

concentration, serum iron, and transferrin saturation than the control group (Table 2). TIBC was significantly elevated in the Fe-deficient rats at wk 2. The pair-fed group had hemoglobin, serum iron, TIBC, and transferrin saturation levels similar to those of the control group, except a lower TIBC level at wk 1 and an elevated serum iron at wk 2, clearly indicating the absence of iron deficiency.

Muscle IscS expression. We cloned the full-length cDNA sequence encoding for rat IscS protein, and this sequence was submitted to GenBank under accession number AF336041. The open reading frame of IscS cDNA contains 1356 nucleotides and potentially encodes a protein of 452 amino acid residues with a calculated molecular mass of 49,720 Da. In rat tissues, the IscS protein of ~47 kDa was immunologically identified only in the mitochondrial fraction. The transcript and protein were present in most rat tissues, predominantly found in heart and muscle, with much lower levels in liver and lung (data not shown).

The expression of mitochondrial IscS protein in the skeletal muscle was significantly affected by diet. It was lower in the Fe-deficient group, and decreased to 45 and 50% of the control levels at wk 1 and 2, respectively (**Fig. 1**A), whereas the control and the pair-fed groups did not differ at each time point. The levels of the IscS mRNA did not differ among the 3 groups at either wk 1 or 2 (Fig. 1*B*). We also measured the specific activity of GDH [nmol/(min · mg protein)], a mitochondrial non-Fe marker enzyme, in the muscle at both wk 1 (C, 53 ± 1; D, 53 ± 8; P, 61 ± 7) and wk 2 (C, 76 ± 4; D, 74 ± 5; P, 71 ± 1), and found no difference among the 3 groups.

Expression of muscle mitochondrial and cytosolic aconitase. The m-aconitase activity was affected by the diets ($P < 0.0001$), and the protein level was affected by diet ($P < 0.0001$) and time ($P < 0.0156$). The activity of m-aconitase was lower in the Fe-deficient group than in the control and the pair-fed groups at both wk 1 and 2 (**Fig. 2**A), decreasing to ~50% of the control levels. In contrast, the pair-fed group had activity similar to that of the control group at both wk 1 and 2. Furthermore, the m-aconitase protein levels in the 3 groups largely paralleled those of m-aconitase activity. The m-aconitase protein levels in the Fe-deficient group decreased significantly to 64 and 58% of the control levels at wk 1 and 2, respectively, whereas the control and the pair-fed groups were not different from each other (Fig. 2*B*).

The c-aconitase activity was significantly affected by diet ($P < 0.0001$), and the protein level was affected by diet ($P < 0.0001$) and time ($P < 0.0107$). The c-aconitase activity in the Fe-deficient group at both wk 1 and 2 was ~45 and 24% of the control levels, respectively (**Fig. 3**A). The c-aconitase protein contents, as represented by the IRE-binding activity of IRP1 in the presence of 2-ME, were 0.764 ± 0.03 and 0.825 ± 0.06 pmol/mg protein at wk 1 and 2 for the control group, and 0.430 ± 0.03 and 0.452 ± 0.07 pmol/mg protein for the Fe-deficient group, respectively. It was significantly lower in the Fe-deficient group than in the control and the pair-fed groups, decreasing to 56 and 55% of the control levels at wk 1 and 2, respectively, whereas there the control and pair-fed groups did not differ from each other (Fig. 3*B*). The protein contents differed in parallel with activity among the 3 groups.

Although they were severely anemic, the Fe-deficient group had the same c-aconitase (**Fig. 4**A) and m-aconitase (Fig. 4*B*) mRNA levels as the other 2 groups. Taken together, our results indicated that iron deficiency modulated muscle c-aconitase and m-aconitase via post-transcriptional regulation.

Correlation between IscS protein content and aconitase activity in muscle. IscS protein content and c-aconitase activity were significantly correlated (**Fig. 5**A, $r = 0.676$, $P = 0.0003$) as were IscS protein content and m-aconitase activity (Fig. 5*B*, $r = 0.764$, $P = 0.0004$).

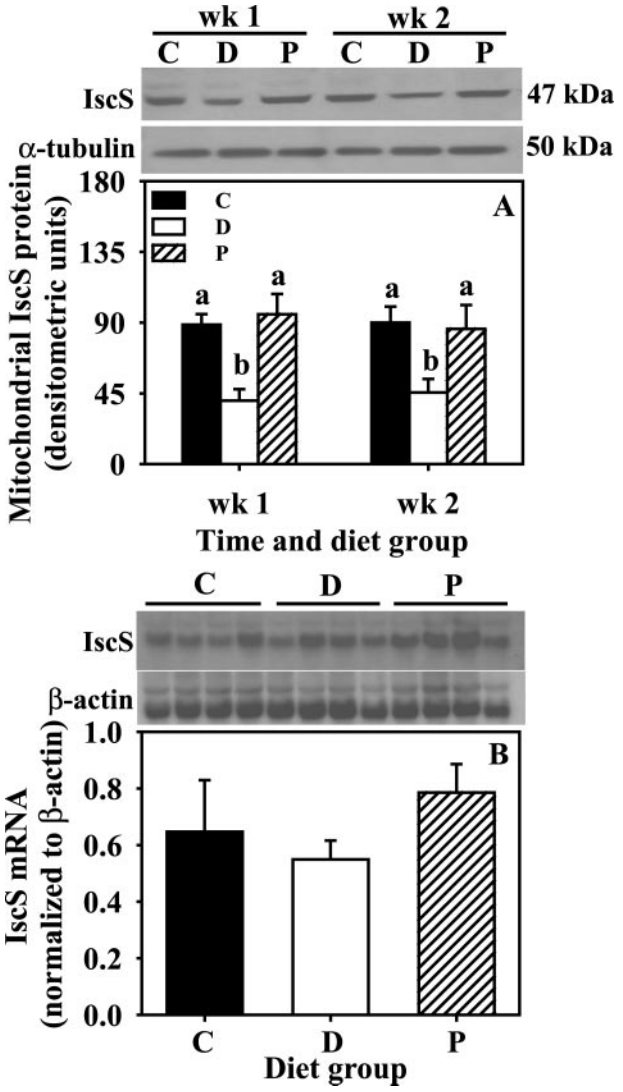

FIGURE 1 IscS protein (*panel A*) and its mRNA (*panel B*) contents in muscle of rats fed Fe-sufficient control diet (C) or Fe-deficient diet (D), or pair-fed the control diet (P). In *panel A*, a representative Western blot for each group was obtained from a pooled mitochondrial protein fraction derived equally from the 4 rats in each group, and α-tubulin was used as loading control. Results are means ± SD, $n = 4$. Bars without a common letter differ, $P < 0.05$. Two-way ANOVA: diet, <0.0001; time, 0.8655; diet × time, 0.0746. In *panel B*, each blot represents 1 rat fed the indicated diet for 2 wk. Results from individual rats were normalized to β-actin and expressed as means ± SD, $n = 4$.

IRE-binding activity in muscle. The IRE-binding activities of IRP1 were 0.07 ± 0.01 and 0.09 ± 0.003 pmol/mg protein at wk 1 and 2 for the control group, and 0.191 ± 0.02 and 0.234 ± 0.05 pmol/mg protein for the Fe-deficient group, respectively. The IRE-binding activities of IRP2 were 0.018 ± 0.004 and 0.016 ± 0.003 pmol/mg protein at wk 1 and 2 for the control group, and 0.043 ± 0.008 and 0.036 ± 0.004 pmol/mg protein for the Fe-deficient group, respectively. The activated IRP1 and IRP2 in the Fe-deficient group were 2.6–2.7 and 2.2–2.3 times the control levels, respectively, whereas no difference existed between the control and the pair-fed groups (**Fig. 6**). In the Fe-deficient rats at wk 2, the IRE-

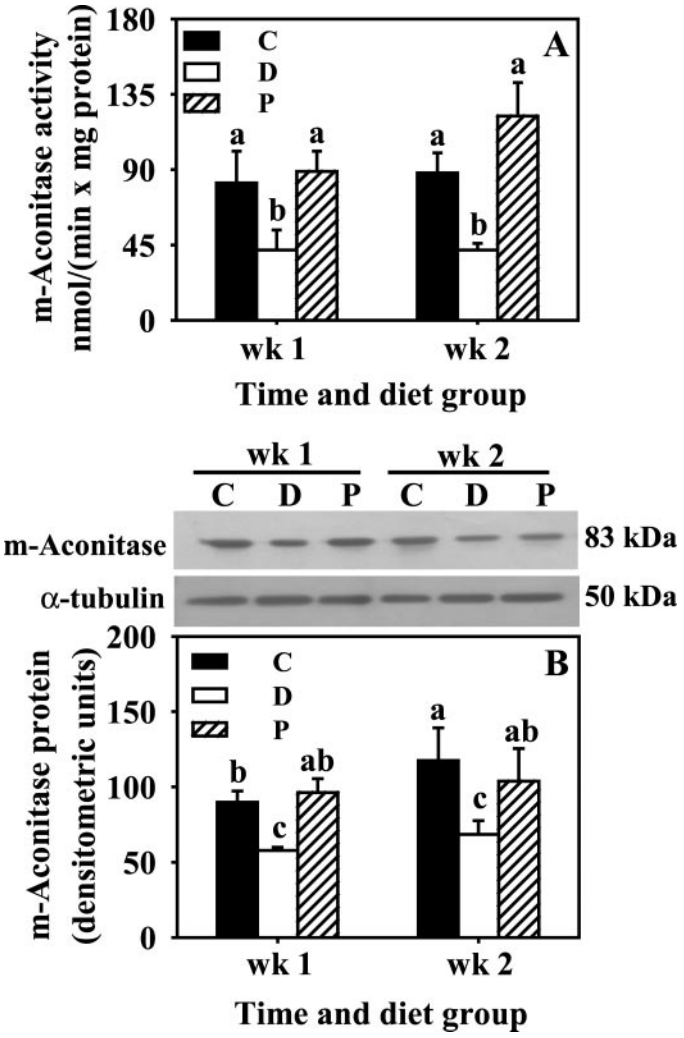

FIGURE 2 Mitochondrial aconitase activity (*panel A*) and its protein level (*panel B*) in muscle of rats fed the Fe-sufficient control diet (C) or the Fe-deficient diet (D), or pair-fed the control diet (P). A representative Western blot for each group was obtained from a pooled mitochondrial protein fraction derived equally from the 4 rats in each group, and α-tubulin was used as loading control. Results are means ± SD, $n = 4$. Bars without a common letter differ, $P < 0.05$. Two-way ANOVA for activity: diet, < 0.0001; time, 0.2403; diet × time, 0.3912; for protein: diet, < 0.0001; time, 0.0156; diet × time, 0.3195.

binding activity of IRP1 was significantly higher than at wk 1 (Fig. 6*A*), whereas that of IRP2 was significantly lower than at wk 1 (Fig. 6*B*). IRE-binding activity and m-aconitase protein content were inversely correlated (Fig. 6*C*, $r = -0.630$, $P = 0.001$).

DISCUSSION

The mitochondrial Fe-S cluster machinery (ISC) is required for the biogenesis of the majority of cellular Fe-S proteins in eukaryotes, including mitochondrial, cytosolic, and nuclear Fe-S proteins (11,43). However, the dependence of extramitochondrial Fe-S proteins on mitochondrial ISC machinery has been shown only in yeast as far as eukaryotes are concerned. The general process of ISC biogenesis is the de novo synthesis of an ISC on some scaffold protein, with sulfur delivered by a sulfur donor. The sulfur-donor IscS is a desulfurase that produces elemental sulfur and alanine from L-cysteine (12–14). Several lines of evidence indicated a multiple cellular localization for this eukaryotic IscS. The protein is located predominantly in mitochondria, yet low amounts are present in the cytosol and nucleus (17,22). In this study, we cloned the cDNA for rat IscS protein and immunologically identified its mitochondrial location. The deduced amino acid sequence shows a relatively high identity of 97% with mouse ortholog (16) and 93% with human ortholog (17), whereas there is only 66% identity with yeast ortholog (15). We could not detect any cytosolic IscS in rat tissues, but the existence of trace amount below the detection limit of our immunoassay is possible and cannot be excluded. The tissue distribution profile identified in rats is similar to that reported for mice (16).

At present, relatively little is known about the in vivo regulation of Fe-S clusters and IscS in mammals. In this study, we demonstrated that the mitochondrial IscS protein in the skeletal muscle decreased specifically (Fig. 1), but its mRNA level remained unaffected by dietary iron deficiency. In a separate study, we compared iron-sufficient and iron-deficient anemic (hemoglobin 30 ± 1 g/L, $n = 4$, data not shown) rats,

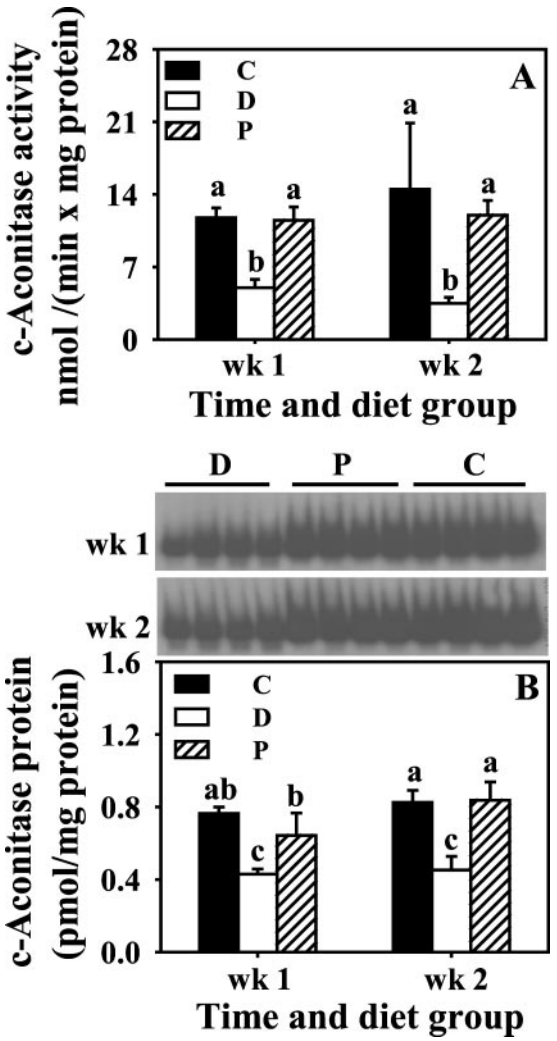

FIGURE 3 Cytosolic aconitase activity (*panel A*) and its protein level (*panel B*) in muscle of rats fed the Fe-sufficient control diet (C) or the Fe-deficient diet (D), or pair-fed the control diet (P). The blot represents ^{32}P-IRE-bound IRP1 from EMSA; 1 lane for 1 rat. Results are mean ± SD, $n = 4$. Bars without a common letter differ, $P < 0.05$. Two-way ANOVA for activity: diet, < 0.0001; time, 0.5454; diet × time, 0.2283; for protein: diet, < 0.0001; time, 0.0107; diet × time, 0.1019.

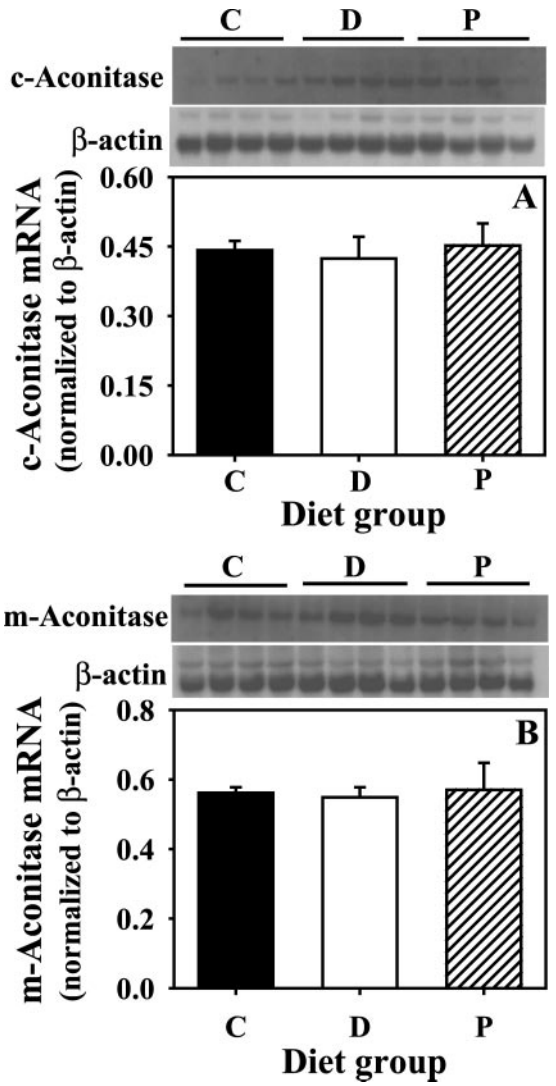

FIGURE 4 Cytosolic and mitochondrial aconitase mRNA levels in muscle of rats fed the Fe-sufficient control diet (C) or the Fe-deficient diet (D), or pair-fed the control diet (P) for 2 wk. The mRNA level of c-aconitase and m-aconitase was detected by Northern blot analysis using 40 and 30 μg of total RNA, respectively. Each Northern blot represents 1 rat. Results from individual rats were normalized to β-actin and expressed as means ± SD, $n = 4$.

and found no difference in the IscS protein content in brain and liver using Western blotting assay. Liver is the main depot for iron storage in the body, and there is little effect of dietary iron deficiency on various iron-protein components of the mitochondrial pathways used for ATP production in liver (25,28,29,44). In contrast, muscle is more susceptible to iron deficiency, and Fe-S enzymes, such as succinate dehydrogenase and NADH dehydrogenase, lose their cofactors and activities in muscle of iron-deficient rats (26,27). This difference between liver and muscle seems to parallel the change of mitochondrial IscS. Therefore, we conclude that there is tissue-specific post-transcriptional regulation of IscS, which is probably associated with the physiological characteristics of different tissues.

Iron regulatory proteins are cytosolic IRE-binding proteins that coordinate cellular iron homeostasis in mammals. Dietary iron intake modulates IRE-binding activity and m-aconitase abundance in rat liver. As dietary iron was decreased to induce iron-deficient anemia (mean hemoglobin 48 g/L) in rats, the IRE-binding activity of both IRP1 and IRP2 increased by 132 and 157%, respectively, whereas the m-aconitase level decreased by 50% (28,29). Similar to the observation for rat liver, the IRE-binding activity of muscle IRP1 and IRP2 increased by 170 and 138%, respectively, in response to iron deficiency in this study. Furthermore, the iron-deficient rats also had a 50% reduction in muscle m-aconitase activity, which was of the same magnitude as the reduction in hepatic m-aconitase. These results indicate that IRE-binding activity and m-aconitase in muscle are modulated by dietary iron via the same molecular mechanisms as their hepatic counterparts. The reduction of m-aconitase activity is attributed to the decreased protein level, which is in turn is regulated via the IRP-IRE interaction because of the presence of a functional IRE in its 5′UTR mRNA (9,10).

We demonstrated a concomitant decline in c-aconitase activity and total IRP1 levels in iron-deficient muscle, which is different from the response of liver or cells. Total IRP1 increases in liver by iron deficiency (28), whereas it remains unchanged in cells regardless of iron status (4,5). Unlike m-aconitase, c-aconitase is not regulated by IRP-IRE interactions, but it is downregulated by iron overload such as hemochromatosis (45), or converted to IRP1 by oxidants produced via metabolism and oxidative stress (6,46,47). Recently, novel eukaryotic factors for cytosolic Fe-S cluster assembly and maturation were identified (48,49). The parallel decline of IscS and total c-aconitase/IRP1 levels in this study suggests a link-

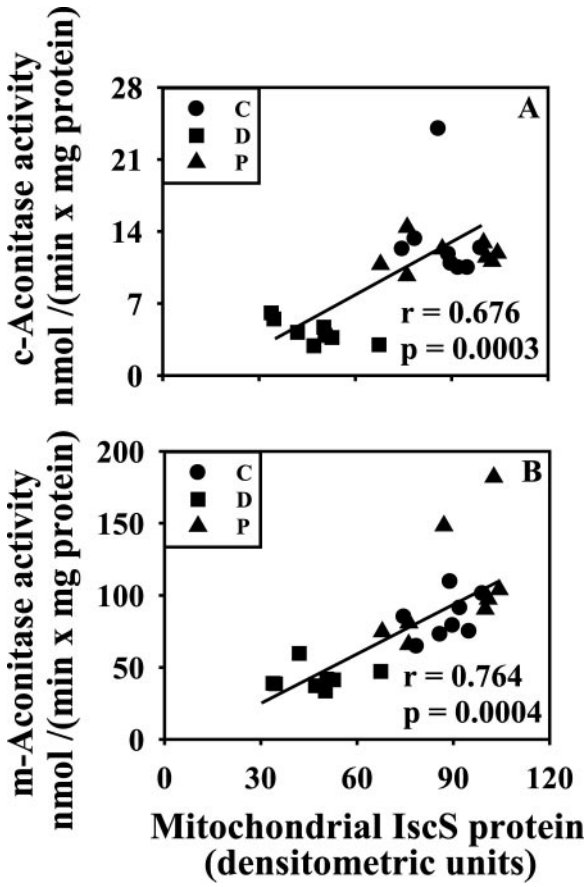

FIGURE 5 Correlation of mitochondrial IscS protein content with the activity of c-aconitase (*panel A*) and m-aconitase (*panel B*) in muscle of rats fed the Fe-sufficient control diet (C) or the Fe-deficient diet (D), or pair-fed the control diet (P); 1 symbol point for 1 rat.

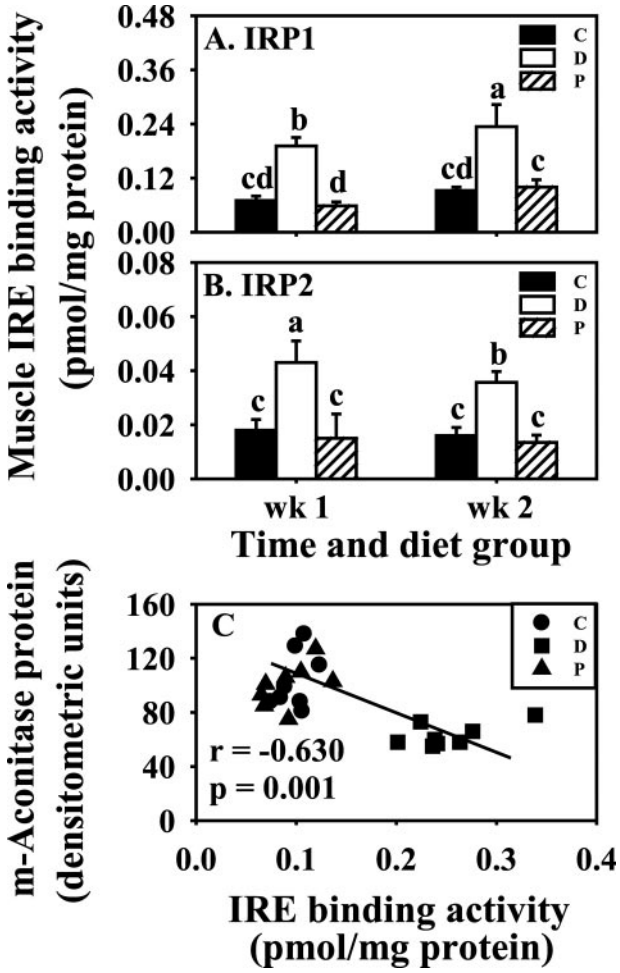

FIGURE 6 The IRE-binding activity of IRP 1 (*panel A*) and IRP2 (*panel B*), and correlation of IRE-binding activity of IRP1 plus IRP2 with m-aconitase protein content (*panel C*) in muscle of rats fed the Fe-sufficient control diet (C) or the Fe-deficient diet (D), or pair-fed the control diet (P). Results are means ± SD, $n = 4$. Bars without a common letter differ, $P < 0.05$. Two-way ANOVA for IRP1: diet, < 0.0001; time, 0.0014; diet × time, 0.6265; for IRP2: diet, < 0.0001; time, 0.0536; diet × time, 0.3343.

ing between the 2. The functional relations among mitochondrial IscS, cytosolic Fe-S machinery, and c-aconitase in mammalian cells await further elucidation.

When muscle (this study) and liver (28,29) are compared, the total c-aconitase/IRP1 level in the liver is 5–10 times higher than in the muscle. However, in rats having similar severity of iron-deficiency anemia, the activated IRP1 levels in the muscle (0.191–0.234 pmol/mg protein) from this study were comparable to the level reported for the liver (0.176 pmol/mg protein) (28). The activated IRP1 constituted 2.8% of the total IRP1 in iron-deficient liver (28), whereas it reached as high as 44–52% in iron-deficient muscle, as demonstrated in this study. Presumably, the skeletal muscle retains a sufficient amount of c-aconitase and activated IRP1 essential for physiological regulation of iron homeostasis.

Furthermore, we speculate that IRP1 apoprotein would be subjected to proteolytic degradation if not protected by IRE binding. If iron deficiency increases only the conversion of c-aconitase to IRP1, the 55% decrease in c-aconitase activity would imply an equivalent increase in the activated IRP1. This level would reach twice as high as the measured or reported values, which would probably overload the cellular IRE pool and disrupt iron homeostasis. Cells had no defense against iron toxicity when expressing a mutant IRP1 that permanently binds IREs regardless of cellular iron status (50). Taken together, the shortage of Fe/S clusters caused by depletion of iron and IscS may prompt a shift toward IRP1 apoproteins and the decline in the total c-aconitase/IRP1 pool.

In summary, our study showed that muscle IRE-binding activity of IRPs and m-aconitase are modulated by dietary iron, and is the first to demonstrate that dietary iron deficiency can cause post-transcriptional downregulation of mitochondrial cysteine desulfurase IscS in the skeletal muscle of rats. Our results also indicate that the total c-aconitase/IRP1 pool shrank in iron-deficient muscle, probably due to a reduced supply of Fe-S clusters caused by decreased mitochondrial IscS.

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