Iron Deficiency Decreases Mitochondrial Aconitase Abundance and Citrate Concentration without Affecting Tricarboxylic Acid Cycle Capacity in Rat Liver$^{1,2}$

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ABSTRACT Mitochondrial aconitase (m-acon) is the tricarboxylic acid (TCA) cycle enzyme that converts citrate to isocitrate. m-Acon mRNA is a potential target for regulation by iron regulatory proteins (IRPs), suggesting a link between dietary iron intake, m-acon synthesis, and energy metabolism. Our previous studies indicate that m-acon is one of a limited number of proteins that is down-regulated in iron-deficient liver. Here we use isolated hepatocytes to study the relationships among decreased m-acon abundance, TCA cycle function and cellular citrate concentration in iron deficiency. Rats were fed an iron-deficient (ID) (2 mg Fe/kg diet) diet, or they were pair-fed (PF) or freely fed (C) a control diet (50 mg Fe/kg diet) for up to 21 d. Hepatocyte total IRP activity was greater by d 2 in the ID group than in the C and PF groups and by d 10, the difference was maximal. Liver IRP activity was inversely correlated with m-acon abundance ($r = -0.93$, $P < 0.0001$). However, the decrease in m-acon abundance did not affect the ability of hepatocytes to oxidize 2-[14C]pyruvate or 1-[14C]acetate, indicating that TCA cycle capacity was not affected. Interestingly, by d 21, total liver citrate concentration was 40% lower in ID than in PF rats, suggesting enhanced utilization of citrate. However, the decrease in citrate concentration was not reflected in a change in liver total lipid concentration. Taken together, our results indicate that the iron-dependent regulation of m-acon in liver does not alter TCA cycle capacity but suggest that IRP-mediated changes in m-acon expression may modulate citrate use in other aspects of intermediary or iron metabolism. J. Nutr. 132: 643–651, 2002.

KEY WORDS: • iron • iron regulatory proteins • mitochondrial aconitase • citrate • tricarboxylic acid cycle • rats

Iron has an essential role in mammalian physiology because iron-containing proteins are required for DNA synthesis, oxygen delivery and use by tissues, as well as for many other physiologic processes. Although iron is an essential nutrient, it can also be toxic. Excess iron can induce formation of toxic oxygen metabolites, which can damage the cell. This is seen in diseases such as hemochromatosis in which excess iron leads to tissue dysfunction due to oxidative damage (1). Because of iron’s essential yet potentially toxic nature, organisms have developed regulatory mechanisms to maintain adequate iron levels. In higher eukaryotes, iron regulatory proteins (IRPs)$^4$

$^1$ Presented in part at Experimental Biology 2001, Orlando, FL [Ross, K. L. & Eisenstein, R.S. (2001) Enhanced activity of liver IRPs in iron deficiency is associated with decreased abundance of mitochondrial aconitase but reduced total liver citrate concentration. FASEB J. 15: A386 (abst)]. These results were reported in part in the ASNS Graduate Student Research Award Competition.

$^2$ Supported in part by the U.S. Department of Agriculture #97–35200–4232 and #01–35200–10683, the National Institutes of Health DK47219, the University of Wisconsin College of Agricultural and Life Sciences Project #3951. K.L.R. was supported in part by the U.S. Department of Agriculture Food and Agricultural Sciences National Needs Graduate Fellowships Program # 94–38420–0781.

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$^4$ Abbreviations used: BSA, bovine serum albumin; C, control; DMT1, divalent metal transporter 1; DNP, 2,4-dinitrophenol; EMSA, electrophoretic mobility shift assay; ETC, electron transport chain; Hb, hemoglobin; ID, iron-deficient; Ig, immunoglobulin; IREG1, iron-regulated transporter; IRE, iron responsive element; IRP, iron regulatory protein; KHB, Krebs-Henseleit buffer; m-acon, mitochondrial aconitase; 2-ME, 2-mercaptoethanol; MTP1, metal transport protein 1; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; PF, pair-fed; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle; TFR, transferrin receptor 1; UTR, untranslated region.

IRPs respond to changes in the intracellular iron level by modulating the synthesis of proteins involved in maintaining iron homeostasis (2–4).

Established targets of IRP action include transferrin receptor 1 (TFR), the plasma membrane protein required for iron uptake, and both subunits of the cytosolic iron storage protein ferritin. IRPs bind specific stem-loop structures, termed iron responsive elements (IREs), located in the untranslated regions (UTRs) of ferritin and TFR mRNA. In iron deficiency, IRPs bind tightly to IREs; conversely IRPs fail to bind IREs in iron-replete conditions. Binding of IRPs to the IRE in the 5’ UTR of ferritin mRNA inhibits its translation, leading to a decrease in iron storage capacity. In contrast, when bound to the IREs in the 3’ UTR of TFR mRNA, IRPs increase the stability of the mRNA, leading to enhanced production of TFR protein. Thus, in iron deficiency, iron storage by ferritin is decreased, and iron uptake into the cell is enhanced for use
in the formation of essential iron proteins. Two recently identified iron transporters, divalent metal transporter 1 (DMT1) and ferroportin 1 (also known as iron-regulated transporter 1 (IREG1) or metal transport protein 1 (MTP1)) have IRE-like elements in their mRNA [reviewed in (3)]. It remains to be determined whether IRPs regulate IREG1 or MTP1 mRNAs.

Two IRPs have been characterized. Under low iron conditions, IRP1 is an IRE-specific RNA binding protein. However, in iron-replete or iron overload conditions, a [4Fe-4S] cluster is assembled in IRP1 converting it to the cytosolic isoform of the tricarboxylic acid (TCA) cycle enzyme aconitase, which catalyzes the interconversion between citrate and isocitrate. Unlike IRP1, IRP2 apparently cannot be converted to an aconitase but instead is degraded when iron levels in the cell are high [reviewed in (5)]. Accordingly, IRPs exhibit reduced binding to mRNAs in iron-replete cells and enhanced binding in iron-depleted cells.

In addition to their presence in mRNA for proteins involved in iron metabolism, IREs or IRE-like elements have been found in mRNAs encoding TCA cycle proteins in some prokaryotes and eukaryotes (6-8). IREs have been characterized in the 5' UTR of the mRNA for the iron-sulfur subunit of succinate dehydrogenase in Drosophila melanogaster and mammalian mitochondrial aconitase (m-acon) (8,9). Both of these IREs were found to be functional in an in vitro protein synthesis system (8,10). We showed that liver m-acon abundance decreases in iron deficiency and that iron regulates m-acon mRNA translation and m-acon synthesis in cultured cells (11-13). Thus, IRPs are believed to regulate m-acon synthesis.

The physiologic role for IRP-mediated regulation of m-acon synthesis has not been elucidated. A number of theories have been proposed. These proposals fall into two categories, those that relate to the function of m-acon in citrate metabolism and/or ATP production and one that has suggested IRPs may modulate the formation of multiple Fe-S proteins in response to changes in iron availability (9,10,14). Recent results suggest that the synthesis of one of the Fe-S components of complex I may be influenced by IRPs in cell culture, although the stem-loop structure in this RNA differs significantly from a canonical IRE (15). However, our previous studies indicate that, in liver, the targets of IRP action (i.e., ferritin, m-acon) selectively respond to iron deficiency, whereas other iron-containing proteins, including complex I, are unaffected (11). Therefore, we have focused our current efforts on determining the extent to which the iron-dependent modulation of m-acon abundance is associated with alterations in TCA cycle function and citrate metabolism. We chose hepatocytes as our model system because m-acon is down-regulated in iron-deficient liver. Furthermore, it is clear that liver has an important role in body fuel metabolism in iron deficiency by maintaining glucose production for use by skeletal muscle, which becomes increasingly reliant on anaerobic production of ATP (16,17).

Our overall goal in this study was to determine the effect of iron deficiency on the relationships among iron status, IRP activity, m-acon abundance and TCA cycle capacity in hepatocytes. We examined the temporal effects of iron deficiency on hepatocyte IRP RNA binding activity and how this relates to changes in m-acon abundance. In addition, we determined the effect of the down-regulation of m-acon in iron-deficient hepatocytes on TCA cycle capacity and cellular citrate concentration. Taken together, our current results indicate that down-regulation of m-acon abundance in iron-deficient liver does not limit TCA cycle capacity and suggests that IRP-mediated changes in m-acon expression are likely to be related to other functions of citrate in intermediary or iron metabolism or to novel roles of m-acon in cellular function.

**MATERIALS AND METHODS**

**Animals and diets.** Weanling male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 42 ± 4 g (n = 66) were housed in individual stainless steel wire-mesh cages in a room with a 12-h light-dark cycle. Upon arrival, rats were fed a purified diet containing 30 mg Fe/kg diet for 4 d (12). After 5 d in our facility, on d 0 of the experimental period, they were randomly divided into one of three treatment groups: iron-depleted group (ID, 2 mg Fe/kg diet, ad libitum intake), an iron-deficient group (ID, 2 mg Fe/kg diet, ad libitum intake), or a pair-fed group (PF, 50 mg Fe/kg diet, fed the mean intake of the ID group). On d 0, 2, 6, 10, 14 and 21, rats were killed between 1000 and 1400 h. All rats had food in their stomach at the time of sacrifice. To ensure the isolation of viable hepatocytes, we prepared cells using one rat per treatment group per day. To obtain four rats per treatment group per time point, the rats were delivered on a 4-d staggered schedule. The use of rats in this study met the standards of the University of Wisconsin Research Animal Resource Center.

**Blood analyses.** Rats were anesthetized with ketamine and xylazine (100 and 10 mg/kg body, respectively). Blood was collected via cardiac puncture after which livers were cannulated for hepatocyte isolation as described below. Hemoglobin (Hb) concentration was determined in heparinized blood by the cyanomethemoglobin method (18).

**Hepatocyte isolation.** Hepatocytes were isolated by a two-step collagenase digestion method (19). The portal vein was cannulated and the liver was perfused in situ with sterile calcium-free Hank's balanced salt solution (Life Technologies, Rockville, MD) containing 0.5 mg/L bovine insulin (Sigma Chemical, St. Louis, MO) and 10 mmol/L cell-culture grade HEPEs (Life Technologies), pH 7.4-7.6. Perfusion buffers were maintained at 37°C and oxygenated with 95% O2/5% CO2. The liver was perfused for 5 min at 15 mL/min to remove blood. Then 15 mL of 10X collagenase buffer (62.5 mmol/L CaCl2 dihydrate; 120 mmol/L NaCl; 10 mmol/L HEPEs, pH 7.4) containing 50 mg collagenase/100 g body (Collagenase A, Roche, Indianapolis, IN) and 20 mg of soybean trypsin inhibitor (Sigma) was added to the remaining perfusion buffer (150 mL). When digestion was complete (~5 min), the liver was placed in a petri dish containing sterile ice-cold Krebs-Henseleit buffer (KHB) containing 2 g/L bovine serum albumin (BSA) Fraction V (Life Technologies) and 1 mg/L insulin. Single cells were obtained by mincing the liver with scissors followed by filtration through a sterile nylon mesh (90-μm pore size, HR Williams, Kansas City, MO). The cell suspension was centrifuged at 40 × g at 4°C in a swinging-bucket rotor for 90 s. The cell pellet was resuspended in KHB and centrifuged again. Viable hepatocytes were then separated from dead cells by centrifugation through Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) (20). The pellet containing the viable cells was resuspended in KHB followed by centrifugation as above. Only cell preparations with at least 90% viability as determined by trypan blue exclusion were used.

**Electrophoretic mobility shift assay (EMSA).** IRP RNA binding activity was determined by EMSA. Cells were lysed in buffer A (21) plus 10 μmol/L MgCl2 (Calbiochem, San Diego, CA), a protease inhibitor. Stock MG-132 (2.5 mmol/L) was in dimethyl sulfoxide. Lysates were centrifuged at 14,000 × g for 6 min and the supernate was stored in liquid nitrogen. Under these conditions, RNA binding activity was stable for at least 4 d (results not shown). Protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). For the RNA binding assay, 10 μg of hepatocyte extract protein was incubated with 1 nmol/L of an internally labeled IRE-containing [32P]RNA composed of the first 70 nucleotides of the rat l-ferritin mRNA (12). RNA binding was quantified by phosphorimaging. The dpm/mg RNA of a [32P]RNA standard curve was determined by scintillation counting and was used to convert digital light units from the phosphorimager into moles RNA. Results are expressed as pmol RNA bound/mg protein.

**Pyruvate and acetate oxidation.** Hepatocyte TCA cycle function was determined by measuring 2-14C]pyruvate (NEN Life Sci-
ence Products, Boston, MA) and [14C]acetate oxidation (American Radiolabeled Chemicals, St. Louis, MO). Pyruvate was used because it is a physiologic substrate for hepatocytes. Acetate oxidation was also examined because it is commonly used to evaluate TCA cycle capacity and it has the advantage of bypassing pyruvate dehydrogenase. Reactions were conducted in triplicate in 25-mL Erlenmeyer flasks closed with serum stoppers. Flasks containing 5 × 10^6 cells in 2 mL media were prewarmed in a 37°C water bath for 30 min. Incubations were initiated by the addition of 1 mL labeled substrate (37°C) in media to give a final specific radioactivity of 55 dpm/nmol. The substrate solution was gassed for 5 min with 95% O2/5% CO2 immediately before use. After 10 min, reactions were terminated by the addition of 2 mL of hexane and 0.5 mL of HClO4 (35 mL/L) to collect CO2 (22), and trapped [14C]CO2 was determined by liquid scintillation counting. Results are expressed as nmol [14C]CO2 produced/(10^6 cells·min). The oxidation reactions were linear for 30 min when 2.5–10 × 10^6 cells were used per reaction (results not shown). Final acetate and pyruvate concentrations of 10 mmol/L were determined to be saturating for normal or iron-deficient hepatocytes (results not shown). Trapping efficiency of [14C]CO2 was 100% as determined using [14C]HCO3 (results not shown).

Hepatocytes were suspended in L-15 medium (Invitrogen, Carlsbad, CA) for acetate oxidation reactions. To measure pyruvate oxidation we used a pyruvate-free Dulbecco’s modified Eagle’s medium because L-15 medium lacking pyruvate could not be obtained. Both media were supplemented with 2 g/L BSA and 1 mg/L insulin. To determine the maximal capacity for oxidation of pyruvate or acetate, we added 1 mmol/L 2,4-dinitrophenol (DNP) to uncouple oxidative phosphorylation.

**Hepatocyte mitochondrial function.** The 3-(4,5-dimethylthiazole-2-y)l-2,5-diphenyl tetrazolium bromide (MTT) assay is a commonly used indicator of mitochondrial function in intact cells in-vitro (23). This assay is based on the reduction of (Pierce) was used. m-Acon protein was quantified using an antibody against bovine heart m-acon that ranged from 4 to 12 ng protein (r = 0.98) [refer to (12) for more details]. The purified m-acon was a kind gift from Dr. Helmut Beinert (University of Wisconsin, Madison) and Dr. Claire Kennedy (Medical College of Wisconsin).

**Liver citrate assay.** Weanling male rats (n = 39) weighing 43.5 ± 0.9 g were fed a purified diet containing 50 mg Fe/kg diet for 3 d (12). After d 4 in our facility, on d 0 of the experimental period, they were randomly divided into one of three treatment groups (C, PF or ID as described above) and fed the iron-deficient or control diet for 0, 7, 14 and 21 d. Rats were anesthetized as described above and the liver was rapidly excised and freeze-clamped in liquid N2, powdered and stored at −80°C. Liver homogenates (100 g/L) were prepared in HClO4 (60 mL/L) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Homogenates were centrifuged at 10,000 × g at 4°C. The supernatant was neutralized with 2 mol/L KOH and centrifuged as described above. Neutralized supernatants were stored at −80°C. Citrate concentration was determined fluorometrically using a coupled enzyme assay (25).

**Liver lipid and triglyceride analysis.** Powdered frozen liver (1 g) was used for determination of total liver lipids using the method of Folch et al. (26). A portion of the final lipid extract was used for colorimetric determination of triglycerides (27).

**Statistical analysis.** The CV was calculated for each analytic procedure and all CVs were <10%. All values are reported as mean ± SEM with n = 4 unless otherwise noted in the figure legends. Differences between group means at individual time points were determined by one-way ANOVA and Least Significant Difference (SAS version 6.11, SAS Institute, Cary, NC). Effect of time and time by group interaction were determined with repeated measures. For all differences, P < 0.05 was considered significant.

**RESULTS**

**Effect of dietary iron intake on growth and blood Hb.** The time-dependent effect of dietary iron intake on weight gain and blood Hb was determined in male Sprague-Dawley rats (Fig. 1). Body weight of rats in groups PF and ID was significantly lower than that of rats in group C by d 5 and 12, respectively (Fig. 1A). When evaluated over the entire experimental period, groups ID and PF gained less weight than group C. At d 21, rats in groups ID and PF weighed 20% less than rats in group C (Fig. 1A). The rate of weight gain over the 21-d experiment and body weight on d 21 did not differ between rats in groups ID and PF. Blood Hb concentration was 107 ± 17 g/L at d 0 (Fig. 1B). Rats fed the iron-deficient diet had lower blood Hb concentrations by d 6 and d 10, compared with groups PF and C, respectively (Fig. 1B). By d 21, blood Hb concentration in group ID was 58 ± 4 g/L, which was

![FIGURE 1](https://academic.oup.com/jn/article-abstract/132/4/643/4687396)  
**FIGURE 1** Time-dependent effect of iron intake on body weight (panel A) and blood hemoglobin concentration (panel B) in rats fed the iron-deficient (group ID) or fed the control diet on a pair-fed (group PF) or ad libitum (group C) basis. For panel A, values are means ± SEM (n = 66) and the arrows indicate the time at which the body weights of rats in group PF (d 5) or group ID (d 12) became significantly different from group C. For panel B, values are means ± SEM, n = 3–6. Different symbols at each time point denote significant differences among groups, P < 0.05.
Effect of dietary iron intake on hepatocyte IRP 1 and IRP 2 RNA binding activity. On d 0, spontaneous [refers to the amount of IRP1 present in the active RNA binding form in the absence of 2-mercaptoethanol (2-ME)] IRP1 RNA binding activity was 0.082 ± 0.015 pmol/mg protein, whereas IRP2 RNA binding activity was 0.045 ± 0.002 pmol/mg protein. By d 2 of the experiment, the spontaneous IRP1 RNA binding activity for group ID was significantly greater, by 35%, than that in groups C and PF (Fig. 2A). The spontaneous IRP1 RNA binding activity continued to increase in hepatocytes from ID rats such that by d 6, it was 79% greater than the activity found in extracts of cells from control rats. By d 14, the IRP1 RNA binding activity in the ID hepatocytes was maximally different (130%) from that measured in cells from rats in the C or PF group. Spontaneous IRP1 RNA binding activity in hepatocyte extracts from groups C and PF did not differ from one another throughout the experiment (Fig. 2A).

The total amount of IRP1 protein can be determined in the presence of high levels of 2-ME, which transiently converts any c-acon into a high affinity RNA binding form (28). Total IRP 1 RNA binding activity, measured in the presence of 2-ME, did not differ among groups at any time point (Fig. 2B). For the ID group, 2-ME-inducible IRP1 RNA binding activity was more than fivefold that of spontaneous IRP 1 RNA binding activity and this difference was eightfold for groups C and PF (Fig. 2B), indicating that compared with groups C and PF, a greater percentage of IRP 1 is in the spontaneous high affinity RNA binding form in group ID.

Like IRP1, hepatocyte IRP2 RNA binding activity increased rapidly and was significantly elevated by d 2 in rats ingesting the ID diet compared with those fed the control diet (Fig. 2C). However, the induction of IRP2 in hepatocytes from ID rats was somewhat more sustained and ultimately more extensive than was the case for IRP1. In hepatocytes isolated from ID rats, spontaneous IRP2 RNA binding activity reached its maximum by d 10 when it was >200% greater than the IRP2 activity in extracts of hepatocytes from rats in groups C and PF (Fig. 2C).

In contrast to the situation at d 0, by d 10, IRP2 RNA binding activity (0.171 ± 0.016 pmol/mg protein) exceeded that measured for IRP1 (0.115 ± 0.009 pmol/mg protein) by 49%. At their maximal degree of activation, iron deficiency resulted in a 44% greater activation of IRP2 compared with IRP1 in hepatocytes. The different kinetics and magnitude of the activation of IRP1 and IRP2 in iron-deficient hepatocytes resulted in a significant shift in the IRP1 to IRP2 ratio from 1.78 at d 0 to 0.75 at d 21.

Combined IRP RNA binding, the sum of the spontaneous activity of IRP1 and IRP2, was significantly increased by d 2 and continued to increase up to d 10 (Fig. 2D). There were no time or treatment effects on the combined RNA binding activity in rats fed the control diet.

Effect of iron deficiency on the steady-state level of hepatocyte m-acon protein. To determine the effect of iron intake on m-acon protein levels, we measured the steady-state abundance of m-acon protein in isolated hepatocytes. On d 0, hepatocytes contained 1.3 ± 0.06 µg of m-acon protein/mg total cell protein (Fig. 3). In rats fed the ID diet, m-acon protein level began to fall by d 6 (P = 0.11) compared with cells from rats fed the control diet. The level of m-acon protein continued to decline such that by d 10 it had reached 44% of that in groups C and PF. Hb concentrations in groups C and PF did not differ at any time point.

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Hepatocyte TCA cycle function is not impaired in liver of iron-deficient rats. To determine the extent to which the decline in m-acon protein affects TCA cycle function, we determined the capacity of hepatocytes to oxidize pyruvate (Fig. 4A) and acetate (Fig. 5). In rats, pyruvate is the more physiologic of the two substrates. We used 2-[14C]pyruvate because production of [14C]CO2 from this substrate will occur only after it has entered the TCA cycle. However, production of [14C]CO2 from this substrate could be indirectly affected by the activity of pyruvate dehydrogenase. Therefore, we also determined the effect of iron deficiency on the capacity of hepatocytes to oxidize 1-[14C]acetate. Acetate is activated to acetyl CoA and directly enters the TCA cycle, bypassing pyruvate dehydrogenase.

The rate of oxidation of a saturating dose of 2-[14C]pyruvate by control hepatocytes at d 0 was 2.0 ± 0.23 nmol [14C]CO2 evolved/(10^6 cells · min) (Fig. 4A). The ability of hepatocytes to oxidize pyruvate did not differ among groups throughout the 21-d experiment (Fig. 4A); there was no significant effect of time or treatment on hepatocyte pyruvate oxidation. The rate of oxidation of 1-[14C]acetate in hepatocytes from C rats on d 0 was 0.41 ± 0.06 nmol [14C]CO2 evolved/(10^6 cells · min), which was ~20% of the rate of oxidation of pyruvate (Fig. 5A). There were no significant effects of time or treatment on hepatocyte acetate oxidation except on d 2 when the value in the ID rats was less than that in C rats (Fig. 5A). However, it was not different from the rate in control hepatocytes at d 0 (P = 0.24, t test).

To measure the maximal capacity of hepatocytes to oxidize a saturating concentration of pyruvate or acetate, we performed the experiment in the presence of the mitochondrial-uncoupling agent DNP. DNP stimulated hepatocyte pyruvate oxidation approximately twofold for all groups (Fig. 4B). At d 14 and 21, the stimulation of pyruvate oxidation by DNP in hepatocytes from group ID was slightly less than twofold. There were no significant effects of time or treatment on the rate of oxidation of pyruvate by hepatocytes treated with DNP, but there was a time-dependent trend (P = 0.082) for the rate to be lower in group ID compared with the other groups (Fig. 4B). At d 21, the mean oxidation of 2-[14C]pyruvate by hepatocytes treated with DNP was 57% less in group ID compared with the other groups (Fig. 4B). In contrast to pyruvate oxidation, DNP decreased 1-[14C]acetate oxidation by almost 50% for all groups compared with the rate of acetate oxidation measured in the absence of DNP (Fig. 5B). Overall, there were no significant effects of dietary iron or time on acetate oxidation measured in the presence of DNP. Furthermore, unlike the situation with pyruvate, there appeared to be no trend suggesting a decrease in the capacity to oxidize acetate in hepatocytes from ID rats.

To further investigate the effect of iron intake on mitochondrial function, we determined the ability of hepatocytes to reduce MTT. MTT serves as an electron acceptor from complexes II/IV of the electron transport chain (ETC), and therefore MTT reduction by intact cells is a measure of the...
activity of the ETC (29). The capacity of hepatocytes to reduce MTT was not affected by dietary iron (Fig. 6). DNP was added to the hepatocytes to determine the effect of dietary iron intake on the maximal capacity of hepatocytes to reduce MTT. In the presence of DNP, MTT reduction increased >40% for all groups compared with that measured in the absence of DNP (results not shown). However, even in the presence of DNP, the ability of hepatocytes to reduce MTT was not affected by iron intake (results not shown).

**Dietary iron deficiency decreases liver citrate abundance.** Because citrate is the substrate for m-acon, we determined whether liver citrate concentrations are affected by dietary iron deficiency. Initially, we measured the steady-state concentration of citrate in liver from a fed and 24-h fasted rat to test whether our assay could detect differences in liver citrate abundance. Similar to previously published results, liver citrate concentrations were lower in a fasted (117 ± 19.6 nmol citrate/g liver) than in a fed rat (152.6 ± 26.5 nmol citrate/g liver).

On d 0, liver citrate concentration was 139.7 ± 33.0 nmol/g (Fig. 7). The total cellular concentration of citrate did not change in the ID rats and at d 21, the citrate concentration in group ID was 154.6 ± 21.9 nmol/g. However, there was a time-dependent increase in liver citrate concentration in rats fed the control diet. Consequently, by d 21, liver citrate was >45% higher in groups C and PF (223.8 ± 17.5 nmol/g, P = 0.07 and 244.9 ± 22.7 nmol/g, P < 0.05, respectively) compared with rats fed the ID diet. Although food intake by group PF was less than by group C, citrate concentrations did not differ in these groups at any time point.

**Effect of dietary iron intake on liver lipid and triglyceride concentrations.** Cytosolic citrate may be used for the de novo synthesis of fatty acids. Interestingly, early studies with iron-deficient rat pups revealed increased liver lipids in iron deficiency (30,31). Therefore we determined whether the decrease in liver citrate concentration observed in the adult iron-deficient rats was associated with increased liver total lipid or triglyceride concentrations. We measured the steady-state concentration of liver total lipids and triglycerides at d 21 in rats from all three dietary treatment groups. The steady-state concentrations of liver total lipid and triglyceride did not differ among the three groups (Table 1).

**DISCUSSION**

Iron regulatory proteins are critical components of a sensory and regulatory network that helps maintain cellular and whole-body iron homeostasis in animals. IRPs influence iron metabolism through their ability to selectively alter the translation or stability of mRNA encoding proteins that modulate the cellular uptake and metabolic fate of iron. In addition to their accepted role in helping to maintain iron homeostasis, IRPs and their bacterial homologs may also influence the synthesis of components of the tricarboxylic acid cycle and/or the electron transport chain (7–13). The enzyme(s) that are targets of this regulation vary among organisms and include mammalian mitochondrial aconitate, *Escherichia coli* aconitate A and B, *D. melanogaster* succinate dehydrogenase and *Bacillus subtilis* cytochrome oxidase, all of which contain an IRE or IRE-like element in their mRNA. Although the purpose of iron-dependent regulation of the synthesis of these enzymes would appear on the surface to be obvious, there is little direct evidence linking iron-related changes in their abundance with alterations in cellular metabolism. To date, mitochondrial aconitate appears to be the only enzyme in the TCA cycle whose mRNA is a target of IRP action in mammals. The overall aim of our current study was to investigate the physiologic consequences of the IRP-dependent regulation of mitochondrial aconitate (m-acon) expression in rat liver on TCA cycle capacity.

Generally speaking, m-acon is not considered to be an enzyme whose activity influences TCA cycle flux. In contrast, citrate synthase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase have been shown to be the enzymes that can be

**TABLE 1**

<table>
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<tr>
<th>Fe</th>
<th>Total lipids (mg/g)</th>
<th>Triglycerides (μmol/g)</th>
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<tr>
<td>Control</td>
<td>31.7 ± 0.86</td>
<td>13.8 ± 1.52</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>24.2 ± 3.09</td>
<td>10.6 ± 1.24</td>
</tr>
<tr>
<td>Fe-deficient</td>
<td>&lt;2</td>
<td>32.3 ± 3.83</td>
</tr>
</tbody>
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1 Values are means ± SEM, n = 4.

2 Liver lipid concentrations were measured for rats that consumed the diets for 21 d.

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**FIGURE 6** Time-dependent effect of dietary intake on rat hepatocyte mitochondrial electron transport chain activity. This graph shows the absorbance of the reduced formazan product from 3-(4,5-dimethylthiazole-2-yI)-2,5-diphenyl tetrazolium bromide (MTT) reduction by isolated hepatocytes in response to diet treatment (as indicated in Fig. 1) as a function of time (see Materials and Methods). Values are means ± SEM, n = 3–5.

**FIGURE 7** Liver citrate abundance in rats fed the iron-deficient (group ID) or fed the control diet on a pair-fed (group PF) or ad libitum (group C) basis. Each time point represents the mean ± SEM, n = 3–4. At each time point, means without a common letter differ, P < 0.05.
regulated to alter TCA cycle flux (32). Unlike m-acon, these three enzymes catalyze reactions that are far from equilibrium in vivo, and such enzymes are classically thought to represent rate-controlling steps in metabolic pathways (33). However, in certain situations, enzymes that catalyze reactions that are near equilibrium in vivo, such as lactate dehydrogenase, can control pathway flux (34). m-Acon is usually considered to catalyze a reaction that is near equilibrium in vivo (34). It is of interest that in certain cell types and in some pathological situations, aconitase activity appears to be a critical factor influencing TCA cycle activity. For instance, prostate epithelial cells are unique in that they produce and secrete large amounts of citrate (35). Interestingly, in these cells, m-acon abundance is hormonally regulated and changes in m-acon expression appear to be the primary factor determining whether citrate accumulates in the cell or is oxidized (36). Similarly, a specific decrease in aconitase activity in cardiac myocytes is associated with impaired TCA cycle activity (37). Finally, previous studies by our laboratory and others have demonstrated that m-acon expression and/or activity is down-regulated in iron-deficient muscle and liver (11,12,38). We demonstrated that the alteration in m-acon expression in liver was part of a highly selective regulation of the targets of IRP action (11). We hypothesized that changes in fuel utilization in liver and skeletal muscle due to iron deficiency are coordinated in part by the IRP regulatory system through iron-dependent regulation of m-acon synthesis. Taken together, the studies outlined above served as an impetus for our current work designed to determine the extent to which the iron-dependent changes in m-acon abundance in rat liver influence TCA cycle capacity.

We used isolated hepatocytes as a tool to determine the effects of decreased liver m-acon abundance induced by dietary iron deficiency on TCA cycle capacity and aspects of citrate metabolism. In hepatocytes, we observed a rapid activation of IRP1 and IRP2 RNA binding activity in response to low dietary iron intake. By d 2, both IRPs were significantly activated in the iron-deficient rats compared with rats fed the iron-adequate diet. In hepatocytes, we found that IRP2 was more extensively activated than was IRP1. These findings are consistent with our previous results using whole liver (11). Ultimately, IRP2 became the predominant contributor to total hepatocyte IRP RNA binding activity in iron-deficient rats, which contrasted with the situation in rats fed the iron-adequate diet. This finding using isolated hepatocytes differs from what we observed in a previous study using whole liver in which IRP2 binding activity exceeded that of IRP1 in rats fed either the iron-deficient or the control diets (11,12). Our current observations indicate that among liver cell types, hepatocytes respond rapidly to alterations in dietary iron intake and they further suggest that cell type-specific differences exist in the relative expression of IRP1 and IRP2 in liver.

In rats fed the ID diet, m-acon abundance was significantly decreased by d 6, whereas in rats fed the control diet, m-acon expression was not altered throughout the experimental period. The extent to which m-acon abundance decreased in hepatocytes from iron-deficient rats was similar to what we observed previously in intact liver (11). Hepatocyte m-acon abundance was strongly and inversely correlated with hepatocyte IRP RNA binding activity. This observation supports the concept that in hepatocytes, m-acon is a target of IRP action and provides further support for the accumulating evidence that the IRE in m-acon mRNA is functional in vivo (9–13). Importantly, our current results indicate that this iron-dependent change in m-acon expression is not induced to alter the rate of oxidation of pyruvate or acetyl CoA. We demonstrated that TCA cycle capacity was not impaired in hepatocytes from iron-deficient rats even when m-acon activity was reduced by 40%. Furthermore, over the 21-d experiment, there was no impairment of the ability to oxidize a saturating dose of 2-[14C]pyruvate or 1-[14C]acetate when hepatocytes from iron-deficient rats were compared with those from rats fed the control diet. Similar results were obtained when these cells were forced to maximize TCA cycle activity through the action of the mitochondrial-uncoupling agent DNP. These findings suggest that the flux through the TCA cycle in vivo was not impaired in iron-deficient hepatocytes. Others have noted defects in aspects of liver mitochondrial function in iron-deficient rats but only after a more prolonged treatment than that used here (31). Nevertheless, our results indicate that there is no apparent relationship between m-acon down-regulation and TCA cycle capacity as a function of iron status of the hepatocytes.

Our results in this and previous studies (11) provide important tests of some of the theories advanced to explain why IRPs may modulate m-acon expression. One hypothesis suggested that IRPs, particularly IRP1, provide a means for controlling oxidative stress due to mitochondrial production of reactive oxygen species (ROS) such as superoxide anion (9). Factors that increase ROS production would activate IRP1, and this could reduce mitochondrial ROS production by repressing m-acon expression and TCA cycle function. It is of interest, therefore, that iron deficiency may cause enhanced production of ROS due to disproportionate loss of iron-containing proteins from the mitochondria during iron deficiency (39). Others have concluded that iron deficiency reduces aerobic production of ATP by reducing m-acon activity (14). However, these studies exposed cultured erythroid cells to a degree of iron deficiency that is not likely to be encountered in mammals in vivo. This fact, coupled with the finding of an apparent iron chelator–dependent change in the activity of several enzymes that are (aconitase) or are not (isocitrate dehydrogenase) known to require iron for their activity, raises a question concerning if such changes represent how an intact animal responds to iron deficiency. In contrast, we found that under physiologic conditions in which hepatocyte IRP RNA binding activity was increased several fold, and m-acon expression was significantly reduced, there was no alteration in TCA cycle capacity. Our results led us to conclude that a larger reduction in m-acon expression is likely to be required to have an effect on TCA cycle flux at least in hepatocytes. A second hypothesis set forth suggested that IRPs mediated the expression of multiple iron-sulfur proteins in relation to iron availability (10). Our previous studies argued against this hypothesis, given our finding that the activity of several iron-enzymes, including two iron sulfur enzyme activities, was not altered in iron-deficient liver even when IRPs were activated several fold (12). Our current results with hepatocytes provide an important functional test of our previous conclusions that were based largely on isolated enzyme assays. Most iron-sulfur enzymes are components of the electron transport chain, and our current findings indicate that any putative decrease in the abundance of these enzymes dictated by IRP action fails to lead to an alteration in the capacity of hepatocytes to oxidize pyruvate or acetate. Finally, recent evidence suggests that m-acon itself may be an RNA binding protein (40). These latest data suggest that like bacterial aconitases, m-acon may bind RNA elements that differ from the classical IRE structure bound by IRP1 and IRP2. This finding raises the possibility that the iron-dependent regulation of m-acon by IRP may represent a means for modulating the use of novel RNA targets by m-acon.
One remaining hypothesis that may explain the iron-de- 
dependent regulation of m-acon expression concerns the poten- 
tial effects of modulating mitochondrial export and cytosolic 
use of citrate. Does down-regulation of m-acon in iron defi- 
ciency lead to enhanced export of citrate to the cytoplasm? 
The increased cytosolic citrate could then be used either in 
exchange metabolism, or in the production of 3-hydroxy-3- 
methylglutaryl-CoA, which is a precursor of cholesterol syn- 

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