Iron Deprivation Induces Transcriptional Regulation of Mitochondrial Biogenesis

Mitochondria are essential organelles that adapt to stress and environmental changes. Among the nutrient signals that affect mitochondrial form and function is iron, whose depletion initiates a rapid and reversible decrease in mitochondrial biogenesis through unclear means. Here we demonstrate that, unlike the canonical iron-induced alterations to transcript stability, loss of iron dampens the transcription of genes encoding mitochondrial proteins with no change to transcript half-life. Using mass spectrometry, we demonstrate that these transcriptional changes are accompanied by dynamic alterations to histone acetylation and methylation levels that are largely reversible upon readministration of iron. Moreover, histone deacetylase inhibition abrogates the decreased histone acetylation observed upon iron deprivation and restores normal transcript levels at genes encoding mitochondrial proteins. Collectively, we demonstrate that deprivation of an essential nutrient induces transcriptional repression of organelar biogenesis involving epigenetic alterations.

Cells must adapt to varying environmental conditions, including changes in the availability of nutrients. In particular, mechanisms controlling mitochondrial content and function, including mitochondrial biogenesis, dynamics, and quality control, are essential for maintaining cellular homeostasis (1–3). Mitochondrial biogenesis involves the coordinated expression of ~1200 proteins encoded from two genomes: nuclear and mitochondrial DNA (4, 5). Dysfunction in mitochondrial biogenesis is associated with a broad range of human diseases, including a number of mitochondrial myopathies, diabetes, and cancer, as well as the aging process itself (6–10). Although important advances have been made in understanding the signals and transcriptional networks that induce mitochondrial biogenesis, many basic features of this process remain obscure. These include epigenetic and posttranscriptional mechanisms that control mitochondrial biogenesis, signals that coordinate gene expression between mitochondria and the nucleus, and states that result in decreased mitochondrial production (11–13). Connecting the disease states and nutritional signals that cause mitochondrial dysfunction to mechanisms of mitochondrial adaptation could offer insights into possible therapeutic interventions and treatments.

Iron is among the most prevalent elements in nature (14). Its ubiquity is apparent in mitochondria, where it can be stored or integrated into cofactors such as heme and iron-sulfur clusters for use in oxidation-reduction reactions throughout the cell (15–17). Throughout the past 60+ years, studies using rat models of iron deficiency have shown that iron deprivation causes a decrease in select mitochondrial proteins, mitochondrial oxygen consumption, and activity of the respiratory chain complexes as well as changes in mitochondrial morphology in a variety of tissues (18). However, apart from several reports on the induction of a mitochondrial-specific autophagy (mitophagy) response (19, 20), the regulation of select mitochondrial transcripts via iron responsive elements (21) and the possible regulation of mitochondrial dynamics (22), little is known about the molecular mechanisms that drive these well known mitochondrial adaptations to iron depletion.

Recently, through the use of matched microarray and mass spectrometry-based proteomic approaches, iron deprivation was found to initiate an adaptive mitochondrial biogenesis response (23). Specifically, iron depletion, through chelation or active transport, caused a rapid and reversible decrease in the abundance of nuclear and mitochondrial transcripts encoding mitochondrial proteins that was equal and opposite in effect size to overexpression of the potent transcriptional co-activator PGC-1α. This response to iron deprivation is dose-dependent, universal across a broad range of mammalian cell types, and independent of well described regulators of mitochondrial biogenesis, including PGC-1α and HIF-1α.

Here we employed biochemical, proteomic, and pharmacological approaches to investigate the mechanism by which iron deprivation causes the rapid and coordinate loss of mitochondrial gene expression in mouse skeletal muscle cells. Utilizing an RNA labeling approach, we determined...
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that the decrease in transcripts is due to a decrease in transcription and not due to increased transcript degradation. Because metazoans lack known iron-responsive transcription factors that could directly explain these results, such as Aft1p and Aft2p in yeast (24), and because of the emerging link between changing metabolite levels and epigenetic modification factors that could directly explain these results, such as iron deprivation—the most common nutritional disorder worldwide (28)—may have both immediate and lasting effects on mitochondrial function.

Experimental Procedures

Cell Culture—Brown preadipocytes were maintained in high-glucose DMEM with 20% FBS and 1× penicillin-streptomycin (Gibco) at 37 °C and 5% CO2. All other cell lines were maintained in high-glucose DMEM with 10% FBS and 1× penicillin-streptomycin (Invitrogen). DFO, DP, and DMOG were obtained from Sigma-Aldrich. SAHA was obtained from Tocris Bioscience.

Relative Quantification Real-time qPCR and EU Labeling—Total RNA was purified from cultured cells using the RNaseasy mini kit (Qiagen). First-strand cDNA was synthesized from purified RNA (500 ng) using the SuperScript III synthesis system for RT-PCR (Invitrogen). Real-time qPCR was performed using SYBR Green-based detection (Applied Biosystems) with Rplp0 as the endogenous control (see the supplemental Experimental Procedures for primer sequences). EU RNA labeling was performed using the Click-iT nascent RNA capture kit (Invitrogen) as detailed in the supplemental Experimental Procedures.

Immunoblotting and Mass Spectrometry—For immunoblot analysis, 2 μg of purified histones, as determined by BCA assay (Thermo Scientific), was separated on a Novex NuPAGE 4–12% Bis-Tris mini gel (Invitrogen), transferred to PVDF, and probed with primary antibodies (listed in the supplemental Experimental Procedures) and a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Invitrogen). Histones were purified from 2 × 106 cells/condition via acid extraction as described previously (29). Mass spectrometry was performed as described previously (30) as detailed in the supplemental Experimental Procedures.

ChIP qPCR—ChIP was performed using ~2 × 106 cells/condition with the Pierce magnetic ChIP kit (Thermo Scientific) following the manufacturer’s protocol. After cross-linking, cell lysis, and nucleus isolation, DNA was digested using 6 μl of micrococcal nuclease (1 units/μl), and nuclei were broken using a sonic dismembrator model 100 or FB-505 microtip probe sonicator (Fisher Scientific). Immunoprecipitations were performed overnight using 5 μg of primary antibody (listed in the supplemental Experimental Procedures) per ChIP reaction. Real-time quantitative PCR was performed using SYBR Green-based detection (Applied Biosystems) (see the supplemental Experimental Procedures for primer sequences). ChIP qPCR data were normalized using the percent input method.

Statistics—p Values were calculated by Student’s two-tailed t test or Welch’s t test as indicated in the figure legends.

Results

Iron Deprivation Decreases Transcription of Nuclear DNA-encoded Mitochondrial Genes—Our previous analyses revealed that iron deprivation initiates an adaptive cellular response involving a global decrease in transcripts encoding mitochondrial proteins and that this effect is especially strong for transcripts encoding subunits of the oxidative phosphorylation (OxPhos) complexes (23). However, the mechanism driving this response has not been determined. From time
course analyses of nuclear-encoded mitochondrial OxPhos transcripts, we found that this decrease starts ~10 h following loss of iron (Fig. 1), suggestive of transcriptional regulation. To directly assess whether the decrease in mitochondrial transcripts was due to a decrease in transcript synthesis or to an increase in transcript degradation, we performed detailed RNA pulse-chase and pulse labeling time course analyses. For the RNA label, we chose the uridine analog 5-ethynyluridine (EU) (31), a non-toxic, widely used, rapid, and specific ribonucleotide label that is incorporated into cellular RNA (but not DNA) with virtually no change to the global transcriptome.

For our pulse-chase analysis, we first labeled cellular RNA for 18 h. Then, after replacing the EU-containing cell culture medium with EU-free medium, we treated the cells with the iron chelator deferoxamine (DFO) and collected them at several time points following treatment (Fig. 2A). We found that DFO did not increase the decay rate of the labeled mRNA for any nuclear DNA (nDNA)-encoded mitochondrial OxPhos transcripts (Fig. 2B) or the non-OxPhos mitochondrial transcript Vdac1 (Fig. 2C) but did increase the half-life of transferrin receptor mRNA (Tfrc) (Fig. 2C), a transcript that is known to be stabilized during iron deprivation (32). DFO treatment also led to an increase in the stability of Hk2 and Slc2a1, which encode proteins involved in glucose metabolism, and whose expression can be influenced post-transcriptionally during nutrient stress via RNA-binding factors (33–35). Importantly, the half-lives of OxPhos transcripts determined from our analyses were similar to those from a global analysis of transcript turnover (36).

Next, we used EU-labeling to examine if iron deprivation causes a decrease in the synthesis of mitochondrial transcripts. For this experiment, we first treated cells with DFO then, 2 h prior to collecting each time point, we added the EU label so that it was incorporated into nascent transcripts made during that stretch of time (Fig. 3A). We found that DFO did not increase the incorporation of the EU label into nDNA-encoded OxPhos transcripts (Fig. 3B) or the non-OxPhos mitochondrial transcript Vdac1 (Fig. 3C) but did increase the half-life of transferrin receptor mRNA (Tfrc) (Fig. 3C), a transcript that is known to be stabilized during iron deprivation (32). DFO treatment also led to an increase in the stability of Hk2 and Slc2a1, which encode proteins involved in glucose metabolism, and whose expression can be influenced post-transcriptionally during nutrient stress via RNA-binding factors (33–35). Importantly, the half-lives of OxPhos transcripts determined from our analyses were similar to those from a global analysis of transcript turnover (36).
mRNAs (Fig. 3B), suggesting a decrease in the synthesis of those transcripts. Synthesis of Vdac1 was also decreased but only at the later time points (Fig. 3C), perhaps explaining why there was no change in its overall levels (23). The syntheses of Tfc, Hk2, and Slc2a1 increased during the time course (Fig. 3C), consistent with their established transcriptional regulation during iron deprivation (37, 38). Interestingly, the change in synthesis of the transcript for Cox4i1, which encodes a subunit of complex IV, was similar to that of the other transcripts; however, its transcript half-life was much greater, potentially explaining why there is no overall change in its total levels (Fig. 1). In contrast to the established iron regulatory protein/iron responsive element system, which controls cellular iron homeostasis through post-transcriptional regulation of gene expression (39), our data indicate that the decrease in nDNA-encoded mitochondrial OxPhos transcripts is due to a decrease in the synthesis of new transcripts, perhaps suggesting the presence of a mammalian iron-responsive transcriptional regulatory program.

Dynamic Changes in Histone Tail Acetylation and Methylation Accompany Cellular Iron Deprivation—The transcriptional effects of iron deprivation are independent of several important transcription factors and transcriptional co-regulators that control mitochondrial biogenesis (23), including PGC-1α and HIF-1α (Fig. 4, A and B). Additionally, as iron-sensing transcription factors, such as Aft1p and Aft2p in Saccharomyces cerevisiae, are seemingly absent in mammalian cells (24) and because of the growing connection between changes in nutrients, metabolism, and the epigenome (25–27), we assessed the potential role for histone PTMs in the mitochondrial response to iron deprivation. To do so, we performed a large-scale, quantitative mass spectrometry-based analysis of histone lysine acetylation and methylation during DFO response and recovery (Fig. 5A). Here we deprived cells of iron for 24 h, took samples at 12 and 24 h, and then passaged the cells into fresh DFO-free medium (Fig. 5A). We continued to passage the cells every day and took samples at days 3 and 5 (Fig. 5A). As described previously (23), 2–4 days following the removal of DFO, OxPhos transcripts returned to the same level as those found in their untreated counterparts, and the non-OxPhos-encoding mitochondrial transcript Vdac1 remained relatively unchanged or was slightly increased during the time course (Fig. 5B). Using a data-independent acquisition mass spectrometry workflow (30), we quantified peptides from nearly 60
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Iron Deprivation Decreases Histone Acetylation at Nuclear DNA-Encoded Mitochondrial Genes—Our data demonstrate that acute iron depletion leads to marked effects on mitochondrial gene expression and the nuclear epigenome. Changes in histone acetylation and methylation can affect transcription through different mechanisms (44). For example, acetylation neutralizes the positive charge of lysine, thereby weakening the interaction between histones and negatively charged DNA. This results in an open chromatin state (euchromatin) and greater access to DNA for transcriptional machinery. Moreover, different types of modifications on different histone lysine residues are correlated with either transcriptional activation or repression. For example, H3K9ac is strongly associated with transcriptional activation and H3K27me3 with repression (45). In both our quantitative MS-based analysis and immunoblot analysis we found an overall decrease in H3K9ac and an increase in H3K27me3 (Fig. 5C and E). To determine whether changes in these modifications are found at nuclear-encoded mitochondrial genes whose expression is affected by iron deprivation, we performed ChIP qPCR analyses. From these experiments, we found reduced levels of H3K9ac at all mitochondrial OxPhos genes analyzed (Fig. 6A), indicating a direct regulatory link between a decrease in histone acetylation and mitochondrial gene expression in response to iron deprivation. We found that the non-OxPhos, nuclear-encoded mitochondrial gene Vdac1, whose expression was unchanged or slightly increased following DFO treatment (Fig. 5B), had relatively minor (and no statistically significant) changes in H3K9ac levels (Fig. 6B) consistent with the delayed DFO-induced decrease in its transcription (Fig. 3C).

FIGURE 4. The mitochondrial transcriptional response to iron deprivation is independent of PGC-1α and HIF-1α. A, level of the indicated mRNAs in wild-type or PGC-1α−/− brown preadipocytes after 100 μM DFO treatment for 24 h. B, level of the indicated mRNAs in wild-type or HIF-1α−/− mouse embryonic fibroblasts after 100 μM DFO treatment for 24 h. Data are displayed as mean ± S.D. of technical triplicate measurements.

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To test whether the change in acetylation is simply a general decrease found at all genes regardless of their expression, we measured the level of H3K9ac at two non-mitochondrial genes, \textit{Hk2} (hexokinase 2) and \textit{Slc2a1} (glucose transporter 1), involved in glucose metabolism and whose expression is transcriptionally increased (Fig. 7A) in response to HIF activation during iron chelation (38). Our ChIP qPCR analysis revealed that iron chelation causes no significant change in histone acetylation at either gene (Fig. 6B), suggesting that the loss of acetylation is specific to certain genes rather than genome-wide. We did not observe an increase in H3K27me3 but, instead, found that this mark was unchanged at mitochondrial genes, suggesting that the changes observed in H3K27me3 are not directly affecting their expression (Fig. 6, A and B). However, it is possible that H3K27me3 affects the expression of those genes through changes in long-range chromatin interactions or regulation of higher-order chromatin structure (46).

To directly examine whether demethylation by the JmjC domain protein family of demethylases is sufficient to drive the loss of mitochondrial transcripts, we treated C2C12 myoblasts with the \(\alpha\)-ketoglutarate mimic dimethyloxalylglycine (DMOG). The JmjC demethylases require iron and \(\alpha\)-ketoglutarate as a cofactor and can be inhibited by \(\alpha\)-keto-glutarate analogs without affecting iron levels (47). DMOG treatment caused increased levels of transcripts encoding glucose metabolic proteins (Fig. 7A), likely via HIF-1\(\alpha\) stabilization (38) or posttranscriptional regulation (33, 35), but did not affect OxPhos-encoding transcripts (Fig. 7B), suggesting that demethylation is not the main regulator of this
response. Cells treated with the structurally and functionally distinct iron chelator 2,2’-dipyridyl (DP) responded comparably to DFO (Fig. 7, A and B).

Pharmacological Inhibition of Histone Deacetylases Blocks the Mitochondrial Biogenesis Iron Deprivation Response—Our results show that the iron deprivation-induced transcriptional changes are accompanied by decreases in histone acetylation at mitochondrial genes. Histone acetylation is regulated by a balance between acetyl-CoA-dependent histone acetyltransferases and the NAD⁺/class III or zinc-dependent (classes I, II, and IV) HDACs (48). To determine whether the effect of iron deprivation on mitochondrial biogenesis is dependent on histone acetylation levels, we treated iron-deprived cells with the clinically relevant HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), which chelates zinc in the active site of class I, II, and IV HDACs but does not inhibit class III HDACs (49). Strikingly, we found that HDAC inhibition completely blocked the effect of iron deprivation on mitochondrial transcripts (Fig. 8A) and histone acetylation levels (Fig. 8B), consistent with histone PTM regulation of gene expression. We also discovered similar effects on mitochondrial transcripts when we treated cells with the HDAC inhibitor trichostatin A (data not shown), which inhibits HDACs through a similar mechanism as SAHA (50).

Taken together, our results reveal that iron deprivation causes reversible changes in histone acetylation and methylation, leading to decreased synthesis of nDNA-encoded mitochondrial transcripts (Fig. 8C).

Discussion

Iron is one of the most abundant elements on earth (14). However, iron deficiency anemia is the most common nutritional disorder in the world, affecting ~25% of the world’s population (28). Iron is an essential cofactor for a wide range of cellular processes, including various metabolic pathways (such as OxPhos), DNA maintenance, and gene expression (51). Dysfunction of iron homeostasis directly causes a variety of human diseases, including Friedreich’s ataxia, and likely contributes to neurodegenerative disease, microbial pathogenesis, various cancers, and cardiovascular disease (52–55). Despite the known association of iron deficiency with these and other disorders, many of the basic mechanisms regulating cellular adaptation to iron deprivation are unknown.

Mitochondria are dynamic organelles that are critical for the survival of nearly every eukaryotic cell type. Iron deprivation is well known to cause extensive changes in mitochondrial function, composition, and structure (18), and genetic perturbation...
of proteins involved in cellular iron metabolism has also been shown to cause severe mitochondrial defects (56–60). In this report, through a blend of unbiased, large-scale mass spectrometry-based approaches and focused, mechanistic experiments, we define an iron deprivation-induced transcriptional response that significantly impacts the expression of genes encoding mitochondrial proteins. Coupling an RNA labeling approach with pulse-chase analyses, we demonstrate that this response is specifically due to a decrease in transcription instead of to an increase in transcript degradation, as might normally be expected for a mammalian iron regulatory program. Further, we show that iron deprivation-induced transcriptional changes are accompanied by dynamic changes in histone acetylation and methylation that are largely reversed when iron levels are restored. Intriguingly, we found that several PTMs did not recover from iron deprivation, suggesting that select epigenetic changes may be persistent and have lasting effects on gene regulatory programs.

Because the enzymes that regulate histone PTMs use central metabolites (such as acetyl-CoA, S-adenosylmethionine, NAD(H), and α-ketoglutarate) and metal ions, they are susceptible to changes in nutrient availability and metabolism that alter the levels of these co-substrates (25–27). Therefore, the observed changes in methylation and acetylation could, for example, be due to iron deprivation-induced reductions in these cofactors and a subsequent reduction in the activity of the iron-O₂-α-ketoglutarate-dependent lysine demethylases or acetyl-CoA-dependent lysine acetyltransferases. In line with this, a reduction in histone acetylation was recently observed in cells depleted of mitochondrial DNA, and genetic restoration of the oxidative tricarboxylic acid cycle and its metabolites reversed this effect (61). Additionally, as part of the mitochondrial unfolded protein response (UPRmt), increased expression of the HDAC SIRT7 and its interaction with the transcription factor NRF1, a key regulator of mitochondrial biogenesis, led to the transcriptional repression of nuclear genes encoding mitochondrial proteins (62).

Due to these expanding connections between histone modifications, mitochondria, and metabolism, we performed ChIP qPCR analyses to discover that acetylation levels are decreased on histones located at nuclear gene bodies that encode mitochondrial proteins, and we reveal that the effect of iron deprivation on transcript levels is blocked by HDAC inhibition, consistent with regulation of nuclear mitochondrial gene expression by histone PTMs. Altogether, our work offers insights into a mammalian iron-regulated transcriptional program and into how nutrient deprivation can drive epigenetic regulation of mitochondrial biogenesis.
Author Contributions—J. W. R. and D. J. P. conceived the project. J. W. R. and K. A. K. designed research, performed experiments, analyzed data, and interpreted results. J. A. D. performed experiments and analyzed data. D. J. P. and J. M. D. directed the project, designed research, and interpreted results. J. W. R. and D. J. P. wrote the paper with help from K. A. K. and review from all authors.

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