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Heme Levels are Increased in Human Failing Hearts

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Abstract

Objectives—The goal of this study was to characterize the regulation of heme and non-heme iron in human failing hearts.

Background—Iron is an essential molecule for cellular physiology, but in excess it facilitates oxidative stress. Mitochondria are the key regulators of iron homeostasis through heme and iron-sulfur cluster synthesis. Since mitochondrial function is depressed in failing hearts and iron accumulation can lead to oxidative stress, we hypothesized that iron regulation may also be impaired in heart failure (HF).

Methods—We measured mitochondrial and cytosolic heme and non-heme iron levels in failing human hearts retrieved during cardiac transplant surgery. In addition, we examined the expression of genes regulating cellular iron homeostasis, heme biosynthetic pathway, and microRNAs that may potentially target iron regulatory networks.

Results—While cytosolic non-heme iron levels were reduced in HF, mitochondrial iron content was maintained. Moreover, we observed a significant increase in heme levels in failing hearts, with corresponding feedback inhibition of the heme synthetic enzymes and no change in heme degradation. The rate-limiting enzyme in heme synthesis, δ -aminolevulinic acid synthase 2 (ALAS2), was significantly upregulated in HF. Overexpression of ALAS2 in H9c2 cardiac myoblasts resulted in increased heme levels, and hypoxia and erythropoietin treatment increased heme production through upregulation of ALAS2. Finally, increased heme levels in cardiac myoblasts were associated with excess production of reactive oxygen species and cell death, suggesting a maladaptive role for increased heme in HF.

Conclusions—Despite global mitochondrial dysfunction, heme levels are maintained above baseline in human failing hearts.

Keywords

heme; ALAS2; mitochondria; iron; heart failure

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INTRODUCTION

Heart failure (HF) rates have soared over the last decade, with almost 6 million Americans affected and the prevalence of the disease rising steadily (1). Molecular mechanisms of HF are complex (2), but mitochondrial dysfunction is an early and common finding in hypertrophied and failing hearts (3). Virtually all aspects of mitochondrial physiology are deranged in HF (4), including mitochondrial number and biogenesis (5), energetic capacity (6), and production of reactive oxygen species (ROS) (7). In addition to generating ATP, mitochondria play a major role in the regulation of iron balance and synthesis of heme and iron-sulfur (Fe/S) clusters (8, 9); however these processes have not been systematically examined in failing hearts.

Indirect evidence points to a link between cellular and mitochondrial iron regulation and HF. Iron is an essential molecule that functions as a cofactor in mitochondrial cytochromes, antioxidant enzymes and more, and its deficiency is associated with cardiomyopathy (10). On the other hand, the redox properties of iron make it an ideal catalyst for the production of toxic hydroxyl radical (OH·) by the Fenton reaction (11), and cardiac dysfunction is a prominent feature of iron overload diseases, such as hemochromatosis (12). Elevated iron levels were noted in the hearts of mice overexpressing the α subunit of the Gq protein, a model of cardiomyopathy (13); however, cellular distribution of iron has not been assessed. Recently, we have shown that accumulation of iron specifically in the mitochondria through a reduction in the levels of ATP-binding cassette transporter B8 (ABCB8), a protein involved in mitochondrial iron export, leads to the development of cardiomyopathy in mice (14). Similarly, aggregation of iron inside the mitochondria has been observed in the hearts of Friedreich's ataxia patients (15), who develop progressive and lethal cardiac dysfunction (16). Thus, maintenance of iron balance inside the heart appears to be critical for its function, but it remains unknown how iron regulation is altered in failing human hearts.

Iron enters the mitochondria through an inner-membrane transporter mitoferrin 2 (Mfrn2) (17, 18), and can either be stored in a complex with mitoferritin (FtMt), or used in heme and Fe/S cluster biosynthetic pathways (19). Heme production begins in the mitochondria with condensation of glycine and succinyl CoA to form δ -aminolevulinic acid (ALA) by the rate-limiting enzyme ALA synthase (ALAS) (9). The next five conversions are carried out in the cytosol. Finally, the synthesis is completed in the mitochondria with insertion of an iron atom into protoporphyrin IX (PPIX) to form heme by ferrochelatase (9) (Figure 1). In the heart, heme functions as a catalytic or structural subunit of mitochondrial electron transport chain (ETC) complexes, myoglobin, antioxidant enzymes and components of the cytochrome p450 (20). Moreover, heme can be broken down by heme oxygenases (HMOX) into elemental iron, carbon monoxide and cardioprotective antioxidant biliverdin (21). Despite the essential role heme plays in the heart, this molecule remains understudied outside of the erythropoietic system.

Fe/S cluster assembly requires over 20 enzymes and scaffolding proteins, and takes place primarily in the mitochondria (8). Fe/S clusters are incorporated into mitochondrial, cytoplasmic and nuclear proteins involved in oxidative phosphorylation, DNA repair, purine metabolism and heme production (22, 23). Disruption of Fe/S cluster synthesis may also lead to cardiomyopathy, as noted in patients with FRDA (24) and mice with deletion of ABCB8 (14), although these conditions are also associated with mitochondrial iron accumulation and ROS.

Given the critical role mitochondria play both in iron homeostasis and in HF, we aimed to characterize the changes in cytosolic and mitochondrial heme and non-heme iron regulation in human failing hearts. Our results demonstrate that both cytosolic and mitochondrial heme

levels are increased in failing hearts, with feedback inhibition of the heme synthetic enzymes, except for ALAS2, which is increased in heart failure and whose expression was previously reported to be restricted to hematopoietic system. We also show that in H9c2 cardiac myoblasts, ALAS2 expression and heme levels are regulated by hypoxia and erythropoietin (EPO), the two pathways that are often altered in failing hearts. Furthermore, we demonstrate that increased heme levels are associated with elevated oxidative stress and loss of viability in cultured cardiomyoblasts.

METHODS

Human samples

Tissue samples were obtained from the tissue bank at Feinberg Cardiovascular Research Institute (Northwestern University) and consisted of samples from non-failing (n=10) and failing ischemic (n=10) human hearts. Failing ischemic tissues were obtained from the explanted hearts of cardiac transplant recipients. Non-failing heart tissue samples were obtained from unmatched organ donors whose hearts were unsuitable for transplantation but had no known cardiac disease. Explanted hearts were immediately placed in cold cardioplegic solution and subsequently frozen in liquid nitrogen. Protocols for tissue procurement were approved by the Institutional Review Board of the Northwestern University. Informed consent was obtained from all transplant patients and from the families of organ donors before tissue collection.

Cell Culture

H9c2 cardiac myoblasts were purchased from ATCC and kept in complete DMEM medium (ATCC) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (P/S). For hypoxic experiments, cells were maintained in a hypoxic chamber at 37°C, 5% CO₂ in the presence of 1% O₂ for up to 8 days. Medium was replaced every two days and cells were collected under hypoxia prior to the analysis. For pharmacologic treatments, cells were grown to 80-90% confluence and incubated with 10 μM hemin (Sigma-Aldrich) and 0.6mg/mL erythropoietin (Sigma-Aldrich) for 48 hours in complete medium.

Mitochondrial fractionation

For tissue samples, we used the Mitochondria Isolation Kit for Tissue (Pierce) according to the manufacturer's dounce homogenization protocol for hard tissue. Homogenized tissue was treated with reagents in the presence of EDTA-free protease inhibitors (Protease Arrest, G-Biosciences). To maintain the integrity of the mitochondria, samples were kept on ice during the isolation process. Following differential centrifugation at 4°C, the supernatant (cytosolic fraction) was collected, and the remaining pellet containing the mitochondria-enriched fraction was dissolved in 1% Triton X-100 (Sigma-Aldrich) in Tris buffered saline (Cellgro).

Non-heme iron measurement

Mitochondrial and cytosolic non-heme iron was quantified using a commercial Iron Assay Kit (BioVision). Briefly, tissue lysate was mixed with acidic solution to release protein-bound iron, followed by a reduction of iron to its ferrous form and incubation with Ferene S compound to produce a colored complex. The absorbance was then measured on Spectra Max Plus microplate reader at 593 nm and normalized to protein concentration of each sample.

Heme iron measurement

For determination of total heme levels, ~5 mg of frozen tissue was homogenized in 1% Triton-X100 in TBS and centrifuged at $5,000 \times g$ for 10 minutes to remove debris. For determination of mitochondrial and cytosolic heme levels, mitochondrial fraction was isolated using Mitochondrial Isolation Kit for Tissue (Pierce) according to the manufacturer's protocol. Protein concentration of cytosolic or mitochondrial lysate was quantified by BCA assay (Pierce, IL) and heme was quantified as described (25). Briefly, equal amounts of protein were mixed with 2 M oxalic acid, heated to 95°C for 30 minutes to release iron from heme and generate fluorescent protoporphyrin IX. Samples were then centrifuged for 10 min at $1,000 \times g$ at 4°C to remove debris; the fluorescence of the supernatant was assessed at 405 nm / 600 nm on Spectra Max Gemini fluorescence microplate reader and normalized to protein concentration of each sample. For determination of unsaturated protoporphyrin IX levels, incubation and heating with oxalic acid steps were omitted. Instead, samples were diluted in PBS, followed by fluorescence measurement and normalization to protein content.

Knockdown and overexpression of ALAS2

For knockdown experiments, ALAS2 and control non-targeting siRNA (Thermo Scientific) were transfected into H9c2 cells using Dharmafect I reagent (Thermo Scientific) for 48 hours according to the manufacturer's protocol. For hypoxia experiments, transfections were repeated every 48 hours to maintain low levels of ALAS2 expression throughout the time course of the study. Knockdown efficiency was confirmed by qRT-PCR and Western blot analyses.

ALAS2 overexpression was achieved through lentiviral transduction. Lentiviral particles coding for ALAS2-GFP fusion protein or GFP-only control were transduced into H9c2 cells at equal multiplicity of infection for 48 hours and overexpression was confirmed by Western blotting.

Quantitative Real-Time PCR

RNA was isolated with RNA STAT-60 (TEL-TEST, Inc), reverse-transcribed with a Random Hexamer (Applied Biosystems), and amplified on a 7500 Fast Real-Time PCR system with SYBR Green PCR Master Mix (Applied Biosystems). Primers were designed using Primer3 (v. 0.4.0) software to target sequences spanning an exon-intron-exon boundary and their specificity was confirmed by running a dissociation curve. mRNA levels were calculated by the comparative threshold cycle method and normalized to β -actin gene.

MitoDNA/genomic DNA

Total DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) and the ratio of mitochondrially-encoded COX I to the genomic 18S gene was determined by quantitative RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems).

Western Blot

~ 5mg of tissue was homogenized in RIPA buffer (Thermo Scientific) in the presence of Protease Arrest protease inhibitors (G-Biosciences), centrifuged at $5,000 \times g$ for 15 minutes to remove debris, and protein concentration of the supernatant determined by BCA assay. Fifteen-30 μg of protein were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes (Invitrogen). The membranes were probed with antibodies against ferritin light and heavy chains (Sigma-Aldrich), HMOX1 (Abcam), ALAS1/2 (Abcam), ferrochelatase (Proteintech), phosphor-JAK2 (Tyr1007/1008), JAK2 (Millipore), NF-E2 (Proteintech), GAPDH (Santa Cruz) and tubulin (Abcam). HRP-conjugated donkey anti-rabbit and donkey

anti-mouse were used as secondary antibodies (Santa Cruz) and visualized by Pierce SuperSignal Chemiluminescent Substrates.

Complex IV activity

Complex IV activity was determined using Complex IV Human Enzyme Activity Microplate Assay Kit (Abcam) according to the manufacturer's protocol. Briefly, complex IV was immobilized in the wells of a 96-well plate by immunocapturing and the specific activity was determined colorimetrically as oxidation of cytochrome c, the substrate of complex IV, by measuring absorbance change at 550 nm for 2 hours at 30°C at 5-min intervals.

Mitochondrial ROS Quantification

MitoSox Red (Invitrogen) was used to assess mitochondrial O₂• production (26). Cells were visualized by microscopy and ROS levels were quantified by ImageJ software. Four fields per each sample were obtained and averaged. Nuclei were counterstained with Hoescht 33342 dye (Invitrogen) and subtracted from the total MitoSox fluorescence to exclude the signal from localization of dye into the nucleus. An accurate overlay between Hoescht and nuclear MitoSox dye was achieved by adjusting microscopy settings prior to data collection, with representative single-channel and overlay images presented in Figure S1A. In addition, total MitoSox fluorescence without nuclear signal subtraction was quantified by ImageJ.

Cell Death Analysis

Following ALAS2 overexpression or hemin treatment, cells were double-labeled with propidium iodide (PI, Sigma-Aldrich, USA) and Alexa Fluor® 350-conjugated Annexin V (Molecular Probes, NY), and analyzed by flow cytometry in a FACS Canto flow cytometer (BD Bioscience). The data are presented as the sum of apoptotic and necrotic cells normalized to the control.

MicroRNA Experiments

Taqman microRNA assays (Applied Biosystems) were used to quantify miR144, 145, 148a, 148b and 152 levels according to the manufacturer protocol. Levels of these microRNAs in failing hearts were quantified by qRT-PCR and normalized to the expression of small RNA U6. To overexpress miR145 we used Pre-miR miRNA Precursors, and to downregulate miR145 we used Anti-miR miRNA Inhibitors (Ambion). Scrambled Pre-miR and anti-scrambled Anti-miR were used in control experiments, respectively.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical significance was assessed with the unpaired Student's t-test; a *P* value of less than 0.05 was considered statistically significant.

RESULTS

Heme is increased in failing hearts

Mitochondria are the primary site of heme and iron-sulfur cluster synthesis, and their function is severely impaired in heart failure (Figure 1). In this study, we characterized changes in cellular and mitochondrial iron homeostasis in failing human hearts. We used left ventricular myocardial tissue from ten patients with terminal heart failure obtained during the heart transplant surgery and ten non-myopathic hearts as controls. Consistent with mitochondrial dysfunction invariably present in HF (27, 28), the mitochondrial DNA copy number and the activity of mitochondrial complex IV were reduced in failing hearts

compared to the controls (Figure S1B,C), thus providing molecular confirmation of the pathology report.

We next measured the levels of non-heme and heme iron in these hearts. There was a significant decrease in cytosolic levels of non-heme iron (Figure 2A) and a trend towards a decrease in the levels of cytosolic iron storage protein ferritin (heavy and light chains, Figure S2A,B). Moreover, mRNA levels of transferrin receptor 1 (TfR1), a protein facilitating cellular iron import, were greatly reduced, while the expression of ferroportin 1 (Fpn1), an iron exporter remained stable (Figure 2B), suggesting that the observed deficiency may be due to a reduction in iron import in cardiomyocytes. However, mitochondrial non-heme iron content was unaltered in failing hearts (Figure 2C), suggesting that the delivery of iron to the mitochondria remains intact in HF.

Heme levels were significantly increased in the cytosolic (Figure 2D) and mitochondrial (Figure 2E) fractions in failing hearts, as determined by the increase in fluorescence of protoporphyrin IX (PPIX) ring, the last synthetic intermediate in the heme synthesis pathway, (25). The observed increase was not due to accumulation of unsaturated PPIX, as the levels of iron-free PPIX were comparable between the two groups (Figure S2C). Thus, even though mitochondrial biogenesis and energetic function are depressed in HF, mitochondrial iron levels (Figure 2F) (obtained by the addition of heme and non-heme mitochondrial iron contents) and heme content in this organelle are increased in the failing hearts.

ALAS2 expression is induced in HF

To understand the mechanism of increased heme levels in HF, we assessed the expression of genes directly involved in breakdown or synthesis of heme. The levels of heme oxygenase 1 and 2 (HMOX1/2), the enzymes catalyzing degradation of heme, were not altered (Figure 3A,B, Figure S3A). These results indicate that the increase in heme levels in HF is not likely to be caused by impaired degradation of heme. Many of the enzymes involved in heme synthesis, including ferrochelatase responsible for iron insertion in PPIX, were actually downregulated in the failing hearts (Figure S3B,C), suggestive of a feedback inhibition on the pathway by high cellular levels of heme as have been previously reported (9). However, mRNA and protein levels of ALAS2, a rate-limiting enzyme catalyzing the first committed step in heme synthesis (29), were significantly upregulated in the failing hearts (Figure 3C,D), providing a potential mechanism for heme accumulation in HF.

The upregulation of ALAS2 in HF is surprising, since the expression of this isoform was previously reported to be restricted to a hematopoietic cell lineage, and it is not known whether upregulation of ALAS2 in the heart can affect heme levels. To test this, we conducted *in vitro* experiments in H9c2 cardiac myoblasts. Lentivirus-driven overexpression of human ALAS2 in H9c2 cells resulted in a ~2-fold increase in its protein levels (Figure 3E). Consistent with our findings in human hearts, heme content was elevated in ALAS2-overexpressing cells (Figure 3F). Moreover, mRNA and protein levels of ALAS1, the non-erythroid isoform of the enzyme, were decreased with ALAS2 overexpression (Figure 3E and S3D), consistent with the feedback inhibition by high intracellular heme content (30).

Regulation of ALAS2 in cardiac cells

Mechanisms for regulation of ALAS2 in hematopoietic cells have been extensively characterized, but it is unknown how ALAS2 is regulated in the heart. The major inducer of ALAS2 in developing erythrocytes is GATA1 transcription factor. However, qRT-PCR analysis revealed no expression of GATA1 in human hearts (qRT-PCR cycle 34.85 ± 0.32). In contrast, protein levels of NF-E2, an erythroid transcription factor that has putative

binding sites in the promoter of ALAS2 (31), were significantly higher in the HF group compared to control (Figure S4A,B).

Since ischemia is common in failing hearts and ALAS2 was shown to be regulated by hypoxia-inducible factor 1 α in erythroid lineage (32), we assessed regulation of ALAS2 by low oxygen tension by subjecting H9c2 cells to 1% O₂ levels. We found ALAS2 expression to be significantly induced by chronic (8-day), but not acute exposure to hypoxia (Figure 4A,B), with a corresponding increase in cellular heme content at day 8 (Figure 4C). Treatment of cells with ALAS2 siRNA during hypoxic incubation effectively ablated an increase in ALAS2 mRNA levels, bringing it down to the normoxic levels (Figure 4D,E), and also prevented the increase in cellular heme (Figure 4F). These data suggest that long-standing ischemia of failing hearts may potentially be responsible for the induction of ALAS2, and provide further evidence that upregulation of ALAS2 in HF may mediate the increase in heme levels.

Finally, ALAS2 is known to be positively regulated by erythropoietin (EPO) in erythroblasts (33) and EPO levels are often elevated in HF patients due to the underlying anemia (34). EPO receptor was previously shown to be expressed in the human heart (35), suggesting that cardiomyocytes may respond to EPO signaling. Western blot analysis of JAK2 kinase, the downstream effector of EPO signaling (36), revealed elevated phosphorylation of this protein consistent with activation of EPO receptor (Figure 5A). Incubation of H9c2 myoblasts with EPO also significantly increased p-JAK2 levels (Figure 5B) and was associated with induction of ALAS2 enzyme on mRNA and protein levels (Figure 5B,C). Heme content was increased in EPO-treated cells (Figure 5D), while suppression of ALAS2 upregulation by EPO using siRNA reversed this increase (Figure 5E,F). Thus, similar to erythropoietic cells, cardiac ALAS2 is responsive to EPO stimulation, which causes an increase in heme levels and may potentially contribute to cardiac heme loading in HF, particularly in the setting of underlying anemia. In summary, we found ALAS2 to be positively regulated by hypoxia and EPO in cardiac cells.

Heme and oxidative stress

Heart failure is characterized by increased oxidative stress, which significantly disrupts myocardial architecture and signaling (37). Free heme has been suggested to exert toxic effects through oxidation of phospholipids, mtDNA damage, and activation of inflammatory response (38). To determine if excess levels of heme contribute to oxidative stress in cardiac myoblasts, we incubated H9c2 cells with hemin and measured mitochondrial ROS production and cell death using MitoSox and propidium iodine staining, respectively. Addition of hemin led to a significant increase in ROS levels (Figure 6A,B, Figure S4C) and was associated with the loss of cell viability (Figure 6C). Overexpression of ALAS2 in H9c2 also induced an increase in ROS (Figure 6D,E, Figure S4D) and was associated with increased cell death (Figure 6F). These data suggest that elevated heme production in HF may be maladaptive through enhancing oxidative stress in these hearts.

MiR-145 does not regulate iron homeostasis in HF

MicroRNA are small non-coding RNA molecules that regulate expression of multiple genes by targeting their 3' untranslated regions (3'UTR) (39). We and others have shown that HF is associated with reduced levels of TfR1, the protein responsible for iron uptake by cardiomyocytes (40). To determine if suppression of TfR1 in HF is mediated by a microRNA, we screened the 3'UTR of TfR1 for putative miR target sequences and the expression of these microRNAs was determined using qRT-PCR (Figure S5A). A significant upregulation of miR-145 was noted in the failing hearts (Figure S5A).

To determine if miR-145 is responsible for the changes in iron homeostasis observed earlier, we overexpressed (Figure S5B) and downregulated (Figure S5C) this miR in H9c2 cardiac myoblasts, followed by the measurement of heme and non-heme iron levels. However, neither of the interventions led to significant changes in heme and non-heme iron content (Figure S5D-G). To determine if miR-145 expression was induced secondarily to the elevated heme levels, we treated H9c2 cells with ALA, the rate-limiting intermediate in heme synthesis pathway, and observed a significant increase in heme levels (Figure S5H), but no change in miR145 expression (Figure S5I). Taken together, these results show that although miR-145 is significantly upregulated in the failing hearts, it does not mediate changes in heme and non-heme iron homeostasis observed in HF and is not regulated by cellular heme levels.

DISCUSSION

The goal of this study was to characterize changes in iron homeostasis occurring in failing human hearts. In spite of mitochondrial dysfunction characteristic of HF, mitochondria-dependent process of heme synthesis was significantly upregulated in failing hearts. We also observed induction of ALAS2, a rate-limiting enzyme in heme synthesis that was previously reported to be expressed exclusively in hematopoietic cells. Moreover, mitochondrial iron levels were maintained at normal levels and were sufficient to support heme synthesis, as we detected very low levels of unsaturated PPIX in the HF group.

It is presently unclear whether the increase in heme levels in HF is an adaptive or maladaptive process. Heme degradation by HMOX1 confers cardiac protection through generation of an antioxidant biliverdin and an anti-inflammatory molecule carbon monoxide (CO) (29). Indeed, induction of HMOX1 was found to be beneficial in HF (41, 42). However, free heme is toxic to the cell as it catalyzes oxidation and breakdown of proteins and DNA, intercalates into lipid bilayers, and damages membrane-bound organelles (38). Similarly, we found that treatment of cardiac myoblasts with hemin significantly elevated ROS generation in the mitochondria and led to a loss in cell viability. Similarly, overexpression of ALAS2, which increases heme content in H9c2 cells, increased both ROS and cell death. On contrary, the levels of HMOX1 were unaltered in the HF samples we examined, suggesting that HMOX1-dependent antioxidation is unlikely to protect the hearts from heme toxicity. Based on the *in vitro* data, we speculate that the induction of heme synthesis in these hearts may be maladaptive through an increase in oxidative stress. Although our data suggests that the use of iron supplementation and stimulants of erythropoietin in anemic heart failure patients, which yielded controversial results (43), may negatively affect cardiomyocytes through induction of ALAS2 and heme synthesis, potential benefits of iron supplementation, as demonstrated before (44), on other organs and hematopoietic system cannot be excluded. It is important to note, however, that our data only provide an association between induction of ALAS2, elevated cardiac heme levels and heart failure. Generation of a cardiac-specific ALAS2-overexpressing mouse model will allow testing this hypothesis directly.

While our experimental design does not identify the mechanism for increased heme synthesis and ALAS2 induction, we hypothesize that chronic ischemia and EPO may play a role. Two isoforms of ALAS enzyme have been identified. ALAS1 is a ubiquitously expressed housekeeping gene and is negatively regulated by heme (45, 46). On contrary, ALAS2 expression is traditionally studied in the context of erythrocyte maturation, where it is induced to stimulate hemoglobinization (47, 48). Unlike other components of heme synthesis pathway, including ALAS1, ALAS2 is not inhibited by high levels of heme, and is positively regulated by hypoxia (32, 49). Our studies show that ALAS2 is expressed in the heart and is significantly upregulated in HF, providing the first evidence of a heart-specific

function for this “non-cardiac” isoform of ALAS. We confirm that, similar to erythroid cells, ALAS2 is regulated by hypoxia in cardiac myoblasts, and that the increase in heme observed in cells subjected to chronic hypoxia is mediated by ALAS2 induction, as knockdown of this protein has also abolished an increase in heme. Moreover, we found that, similar to developing erythrocytes, EPO stimulation induced ALAS2 expression and subsequently elevated heme levels. The significance of these findings in the context of failing hearts remains to be determined. Despite elevated EPO levels, HF patients were found to be resistant to EPO signaling in promoting hematopoiesis (34), but it remains unknown whether the sensitivity of cardiomyocytes to EPO stimulation is also altered.

In addition to showing an increase in heme content in HF, we find that miR-145 is strongly induced in the failing human hearts, but it does not mediate the changes in heme that were observed in HF. MiR-145 is known to play a role in smooth muscle differentiation (50, 51) and also exhibits tumor suppressor activity (52, 53). To the best of our knowledge, this is the first report of miR145 upregulation in heart failure and, naturally, it would be of interest to see if this induction has any functional consequences.

We show that mitochondrial iron levels are increased in the failing hearts due to an increase in heme content, suggesting that mitochondrial iron homeostasis is maintained in spite of severe mitochondrial dysfunction. We would like to note, however, that preservation of mitochondrial iron content does not necessarily imply that all iron-dependent processes remain unaffected. Specifically, it remains to be determined how HF affects the process of Fe/S cluster assembly in the mitochondria and their incorporation into proteins. Fe/S clusters are essential for maintenance of normal energy homeostasis, as they are required for the function of several subunits of electron transport chain (ETC) complexes and the tricarboxylic cycle enzyme aconitase. In fact, we and others report reduced activity of ETC complexes and aconitase in HF, suggesting that Fe/S cluster biosynthesis may be disrupted in failing hearts (54).

Finally, the potential limitations of our studies include the lack of data on demographics, underlying cause of heart failure, and/or medical therapy received by patients prior to the transplant surgery and retrieval of the heart. Likely, our samples come from a heterogeneous patient population. The effects of medications, methods for preservation, and ischemic time for both control and failing hearts are important factors that may have impacted the reported findings. Finally, the mechanisms of cellular iron regulation are complex and multiple. Thus, unmeasured confounders may potentially contribute to the presented findings. Despite these limitations we nevertheless observe a consistent and robust increase in cardiac heme levels in failing hearts. Thus, we can speculate that induction of heme synthesis pathway represents a common and important pathophysiologic finding in HF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

ALA	δ -aminolevulinic acid
ALAS2	ALA synthase 2
ABCB10	ATP-binding cassette B10
ETC	electron transport chain
EPO	erythropoietin
Fe/S	iron-sulfur
HF	heart failure
HMOX1	heme oxygenase 1
Mfrn2	mitoferrin 2
FtMt	mitochondrial ferritin
PPIX	protoporphyrin IX
ROS	reactive oxygen species

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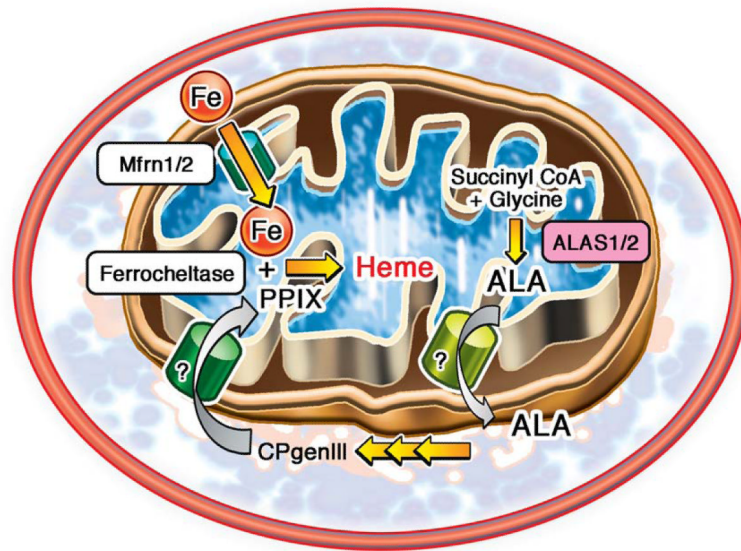


Figure 1.
Schematic representation of cellular heme synthesis pathway

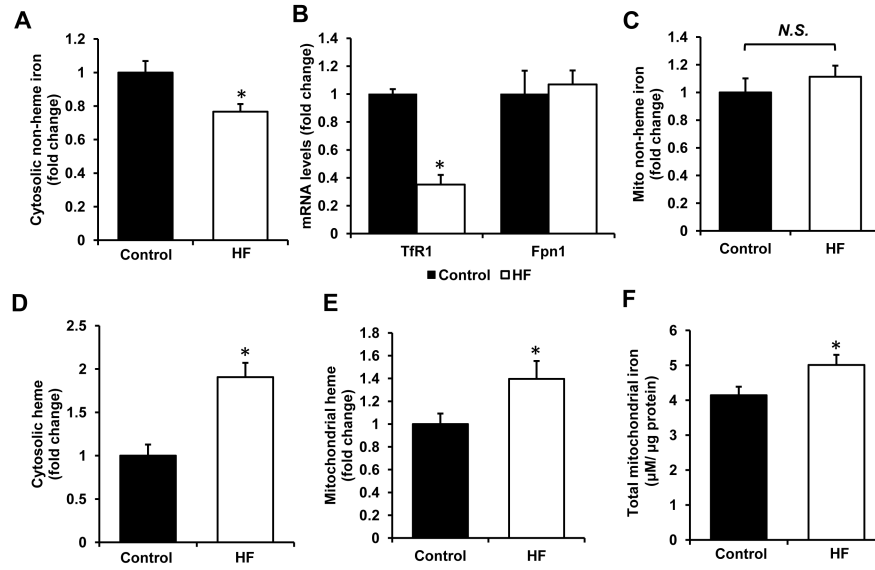


Figure 2. Heme and non-heme iron regulation in HF

(A) Cytosolic non-heme iron levels in control and failing hearts normalized to the cytosolic protein concentration (n=10). (B) mRNA expression of TfR1, an iron importer, and Fpn1, an iron exporter, in HF and control groups (n=10). (C) Non-heme iron levels in the mitochondria of control and failing hearts normalized to mitochondrial protein content (n=10). Cytosolic (D) and mitochondrial (E) heme levels in control and failing hearts normalized to the protein concentration of cytosolic and mitochondrial fractions, respectively (n=10). (F) Total mitochondrial iron levels, obtained by addition of non-heme and heme iron content (n=10). Data are presented as mean \pm SEM. * $p < 0.05$ vs. control.

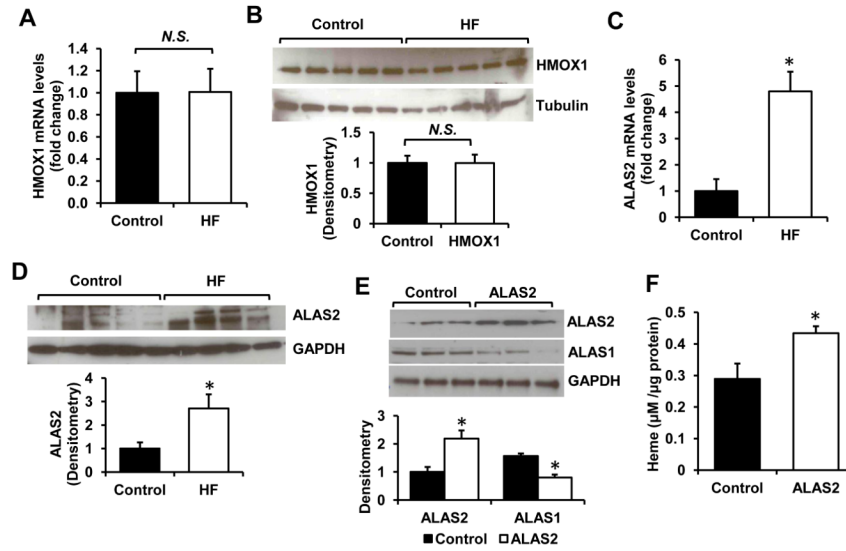


Figure 3. ALAS2 is upregulated in failing hearts

mRNA (A) and protein (B) levels of HMOX1 in control and failing hearts (n=5-6). mRNA (C) and protein levels (D) of ALAS2 in control and failing hearts (n=4-6). (E) Western blot analysis of ALAS1/2 proteins in H9c2 with lentiviral overexpression of ALAS2 enzyme (n=3). Densitometry analyses are presented below the Western blots. (F) Heme levels in H9c2 cells transfected with ALAS2 or control lentiviral vector (n=6). Data are presented as mean \pm SEM. * $p < 0.05$ vs. control.

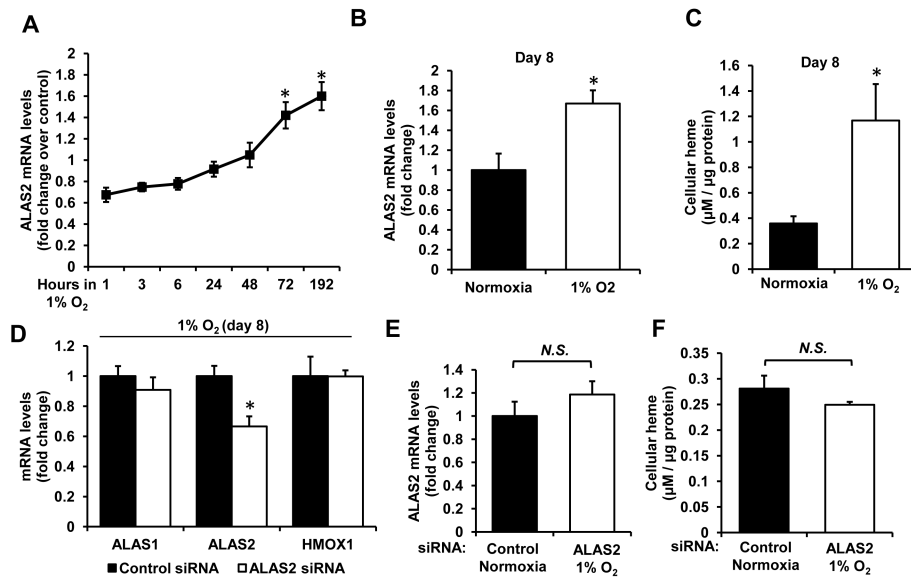


Figure 4. Regulation of ALAS2 by hypoxia in cardiac myoblasts

(A) Time-course of ALAS2 mRNA expression in H9c2 cardiac myoblasts subjected to hypoxia (n=6). (B) ALAS2 mRNA levels after 8 days in hypoxia or normoxia (n=6). (C) Cellular heme content after 8 days in hypoxia or normoxia (n=6). (D) mRNA levels of heme synthesis/degradation enzymes in hypoxic cardiac myoblasts with or without siRNA-mediated ALAS2 knockdown (n=6). (E) Comparison of ALAS2 mRNA levels in control normoxic and ALAS2 siRNA-treated hypoxic cells after 8 days (n=6). (F) Heme levels in control normoxic and ALAS2 siRNA hypoxic cells after 8 days (n=6). Data are presented as mean \pm SEM. * $p < 0.05$ vs. control.

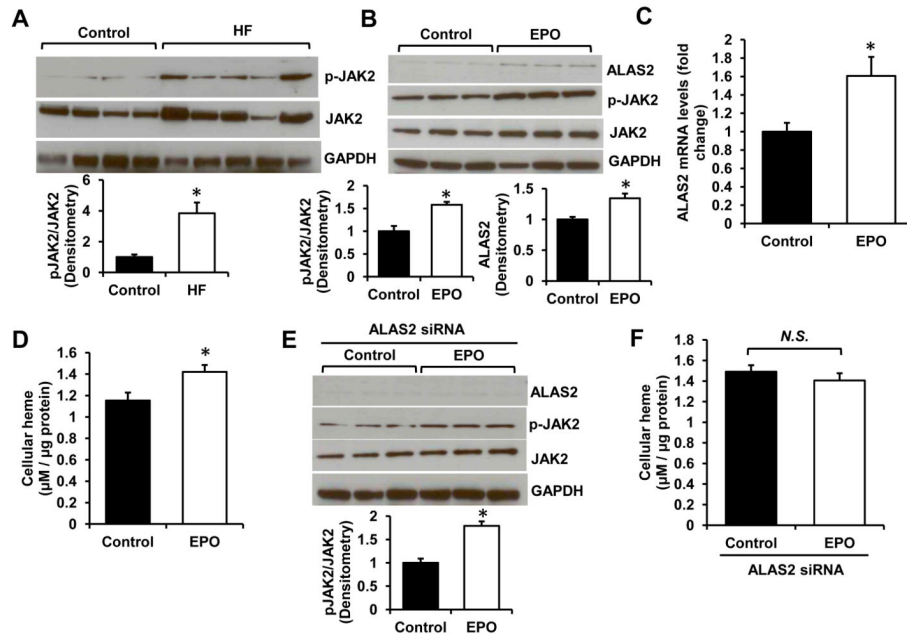


Figure 5. ALAS2 is regulated by erythropoietin

(A) Western blot analysis of p-JAK2 and JAK2 protein in failing and control hearts (n=4-5). (B) ALAS2 and pJAK2/JAK2 protein levels in H9c2 cells treated with 0.6mg/mL EPO (n=3). (C) qRT-PCR analysis of ALAS2 mRNA expression in H9c2 cells with EPO or vehicle control treatment (n=6). (D) Cellular heme content with EPO treatment in cardiac myoblasts (n=6). (E) Western blot analysis of ALAS2 and pJAK2/JAK2 in ALAS2 siRNA-treated cells incubated with EPO or vehicle control (n=3). (F) Cellular heme content in EPO- or vehicle-treated cells with ALAS2 siRNA knockdown (n=6). Data are presented as mean ± SEM. * p<0.05 vs. control.

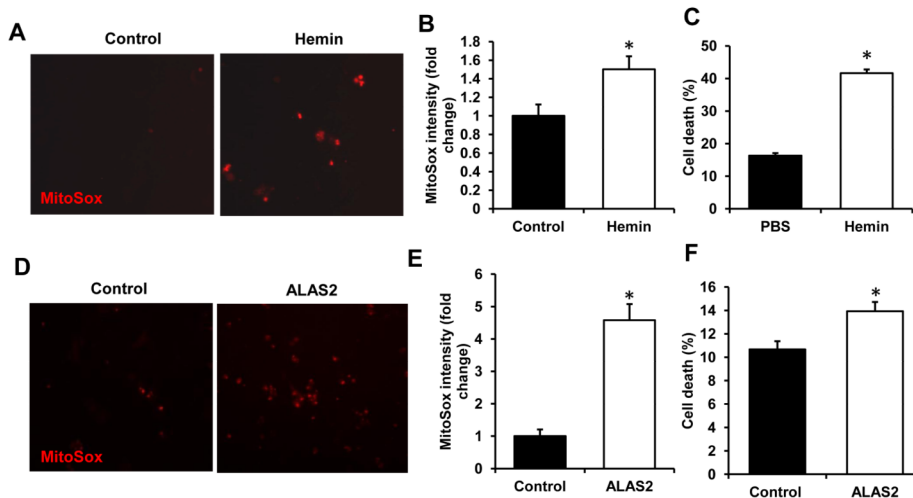


Figure 6. Heme and ALAS2 overexpression increase oxidative stress

(A) Representative images of H9c2 incubated with the vehicle control or 10 μ M hemin for 6 hours and stained with mitochondria-specific ROS-sensitive dye MitoSox. (B) Image J analysis of MitoSox-stained H9c2 following the treatment with vehicle or hemin (n=4-5, 4 fields per sample). (C) Cell death assessed by propidium iodide/Annexin V double-labeling with hemin treatment as in A (n=6). (D) Representative MitoSox images of H9c2 with or without ALAS2 overexpression. (E) Quantification of MitoSox staining with ALAS2 overexpression (n=4-5, 4 fields per sample). (F) Cell death with ALAS2 overexpression assessed as in C (n=6). Data are presented as mean \pm SEM. * p<0.05 vs. control.