Cardioprotective HIF-1α-frataxin signaling against ischemia-reperfusion injury

Gayani Nanayakkara,1* Abdullah Alasmari,1* Shravanthi Mouli,1 Haitham Eldoumani,1 John Quindry,2 Graham McGinnis,2 Xiaoyu Fu,1 Avery Berlin,1 Bridget Peters,2 Juming Zhong,3 and Rajesh Amin1

1Cardio-Metabolic Lab, Department of Drug Discovery and Development, Harrison School of Pharmacy, Auburn University, Auburn, Alabama; 2School of Kinesiology, Auburn University, Auburn, Alabama; and 3Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, Alabama

Submitted 5 December 2014; accepted in final form 1 May 2015

Previous studies have demonstrated the protective signaling of hypoxia-inducible factor (HIF)-1α against ischemia-reperfusion (I/R) injury in the heart. In the present study, we provide further evidence for a cardioprotective mechanism by HIF-1α against I/R injury exerted via the mitochondrial protein frataxin, which regulates mitochondrial Fe-S cluster formation. Disruption of frataxin has been found to induce mitochondrial iron overload and subsequent ROS production. We observed that frataxin expression was elevated in mice hearts subjected to I/R injury, and this response was blunted in cardiomyocyte-specific HIF-1α knockout (KO) mice. Furthermore, these HIF-1α KO mice sustained extensive cardiac damage from I/R injury compared with control mice. Similarly, reduction of HIF-1α by RNA inhibition resulted in an attenuation of frataxin expression in response to hypoxia in H9C2 cardiomyocytes. Therefore, we postulated that HIF-1α transcriptionally regulates frataxin expression in response to hypoxia and offers a cardioprotective mechanism against ischemic injury. Our promoter activity and chromatin immunoprecipitation assays confirmed the presence of a functional hypoxia response element in the frataxin promoter. Our data also suggest that increased frataxin mitigated mitochondrial iron overload and subsequent ROS production, thus preserving mitochondrial membrane integrity and viability of cardiomyocytes. We postulate that frataxin may exert its beneficial effects by acting as an iron storage protein under hypoxia and subsequently facilitates the maintenance of mitochondrial membrane potential and promotes cell survival. The findings from our study revealed that HIF-1α-frataxin signaling promotes a protective mechanism against hypoxic/ischemic stress.

NEws & NoteWorthy

The present study provides evidence for a cardioprotective transcriptional regulatory mechanism by hypoxia-inducible factor-1α of the mitochondrial protein frataxin against ischemia-reperfusion injury. Frataxin regulates mitochondrial Fe-S cluster formation and protects against mitochondrial iron overload, the subsequent ROS production, and myocardial energy dysregulation.

Despite recent therapeutic advances, coronary heart diseases, including acute myocardial infarction (MI), are the most common forms of cardiovascular diseases, which account for high mortality rates in the United States. Prognosis and mortality rates from MI depend on the infarction size, which also determines the rate of progression to heart failure (31). Therefore, understanding the molecular signaling cascades that occur in the myocardium due to MI is necessary to identify novel therapeutic targets to mitigate the cardiac damage and progression to heart failure.

Frataxin is a highly conserved nuclear encoded mitochondrial protein that is abundantly expressed in tissues with a high metabolic rate, such as the heart, neurons, kidney, and liver (18). Defects in frataxin production in Friedreich’s ataxia (FRDA), which is an autosomal recessive genetic disorder, produce complications in cardiac and neuronal tissues by disrupting cellular homeostasis (5). FRDA is manifested due to a homozygous unstable GAA triplet repeat expansion in the first intron of the frataxin gene. This expansion leads to abnormal DNA structures that interfere with frataxin transcription, leading to a significant reduction of frataxin levels (2). The length of the GAA expansion is proportional to the degree of frataxin deficiency in individuals with FRDA. Moreover, cardiomyopathy arises in patients with larger expansion, and it does not depend on the duration of the disease (30).

The exact function of frataxin has not been fully elucidated; however, it has been found to play a role in iron homeostasis by acting as a chaperone during Fe-S cluster formation. These Fe-S clusters act as cofactors and facilitate the normal enzymatic functions that regulate oxidative phosphorylation (43). Additionally, frataxin acts as an iron storage protein during conditions of iron overload (32). Therefore, it has been suggested that frataxin offers a vital antioxidant mechanism by attenuating Fenton’s reaction, which produces ROS during an iron surplus (8).

How frataxin levels are regulated under physiological and pathological conditions are not fully understood. However, frataxin expression has been observed to be upregulated in several tumor cell lines in response to hypoxic stress and promotes tumor cell survival and progression. It has also been suggested that hypoxia-inducible factor (HIF)-1α may play a significant role in the regulation of frataxin expression in response to hypoxia (9). HIF-1α is a heterodimer comprised of α- and β-subunits and is considered as the master transcription factor that regulates the hypoxic response during an ischemic event (20). The activity of HIF-1α is measured by the level of expression and the activity of HIF-1α subunit. Under normoxic conditions, HIF-1α expression is tightly regulated by ubiquitination and subsequent proteasomal degradation (13). These regulations are suppressed under hypoxia, which leads to elevated expression levels of active HIF-1α that translocate to the nucleus and transcriptionally regulate many genes asso-
iated with physiological processes, including angiogenesis and metabolism (16). Recently, we observed that frataxin expression is significantly increased in response to hypoxia in cardiomyocytes as well as in hearts subjected to ischemia-reperfusion (I/R) injury.

Therefore, in the present study, we tested the hypothesis that HIF-1α induces a protective mechanism in response to hypoxia in cardiomyocytes by transcriptionally activating frataxin. We constructed a cardiomyocyte-specific HIF-1α knockout (KO) mouse model and also frataxin overexpressed/knockdown (FXN-OE) H9C2 cardiomyocyte cell lines to validate the protective mechanism of the HIF-1α-frataxin signaling pathway. We observed that attenuation of HIF-1α activity significantly suppressed frataxin expression in response to hypoxia/ischemia in both in vitro and in vivo models. Our data suggested that elevated levels of frataxin under hypoxic/ischemic stress prevent mitochondrial iron accumulation and subsequent ROS production, thus help to maintain mitochondrial integrity in cardiomyocytes. The present study reveals a novel cardio-protective signaling pathway involving frataxin that is regulated by HIF-1α and exerts protection and maintains cell viability under hypoxic/ischemic stress in cardiomyocytes.

MATERIALS AND METHODS

Animals. The experimental and breeding protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Auburn University. Animals were housed in Auburn University’s Biological Research Facility in a controlled environment (23°C, 12:12-h light-dark cycle) with free access to water and standard chow diet. Cardiomyocyte-specific HIF-1α KO mice were constructed using a Cre-loxP system. Transgenic mice with HIF-1α flanked with loxP sites (stock no. 011038) and mice with Cre-recombinase expression driven by the α-myosin heavy chain promoter (stock no. 007561) were purchased from Jackson Laboratories. loxP mice and Cre mice were mated, and their offspring was genotyped by PCR using genomic DNA isolated from tail snips to identify mice with heterozygous HIF-1α deficiency. Heterozygous HIF-1α KO mice were backcrossed with loxP mice to obtain cardiac-specific HIF-1α KO mice. Both Cre and loxP male mice were used as controls for I/R surgeries, and results are shown in Fig. 1.

I/R surgery. I/R surgeries were performed on 12-wk-old male HIF-1α KO mice as well as on loxP and Cre mice used as controls. Briefly, mice were anesthetized with pentobarbital sodium and ventilated mechanically. Hearts were exposed by a left thoracotomy, and the proximal left anterior descending coronary artery was reversibly ligated for 30 min with an 8.0 silk suture mounted on a tapered needle. Reperfusion was achieved and completed by loosening the ligature for 2 h. Sham-operated mice underwent a time equivalent procedure in the absence of left anterior descending coronary artery occlusion.

The area at risk was visualized by injecting Evans blue dye into the femoral vein. Hearts were enucleated, and sections were then taken. Infarction size was determined by incubating the heart slices in 1% triphenyltetrazolium chloride (1% solution). Heart sections were then imaged by a Nikon dissecting microscope. The infarcted and noninfarcted regions were then separated and snap frozen for further analysis.

Chemical hypoxia in mice. Mice were injected intraperitoneal with 1 mg dimethylxalolylglycine (DMOG; Cayman Chemical) in saline 2 h before mice were euthanized.

Cell culture. H9C2 ventricular rat embryonic cardiomyocytes were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were maintained at 37°C and 5% CO2 levels with humidity. Hypoxia was achieved by exposing the cells to 1% O2 gas in an airtight Billups Rothernberg hypoxia chamber with adequate humidity at 37°C for 18 h. Chemical hypoxia was induced by treating the cells with DMOG (1 mM). HIF-1α was inhibited using KC7F2 (40 μM, Cayman Chemicals) and an RNA inhibition (RNAi) system specific for HIF-1α.

HIF-1α RNAi. Plasmids for HIF-1α RNAi were kind gifts from Connie Cepko’s laboratory and purchased from Addgene (plasmid nos. 21103 and 21104) (4). Cells were transfected with increasing concentrations of plasmid using Jetprime transfecting reagent (Polyplus). HIF1α RNAi target sequences for plasmids were as follows: 5’-GAGCTTGCTCATCAGTTGCCA-3’ (plasmid no. 21103) and 5’-GGTTGAAACTCAAGCAACTG-3’ (plasmid no. 21104).

Construction of FXN-OE H9C2 cardiomyocytes. The FXN-OE cardiomyocyte cell line was generated using a lentivirus constructed by the gateway system. Briefly, cDNA of frataxin was cloned into a gateway donor vector, which was subsequently cloned into a pLX 302 lentiviral mammalian expression destination vector using LR clonase. The pLX 302 vector consists of a puromycin-selectable marker. The vectors were then transformed into DH5α competent bacteria, and the plasmids were isolated using the Promega’s Pure Yields Plasmid Midi kit. The purified plasmid was cotransfected with a lentiviral packaging vector (psPAX2) and envelope vector (pMD2.G) into a human embryonic kidney (HEK)-293T cell line. Media with the virus was extracted after the third day of transfection and filtered using a 0.45-μm syringe filter. Purified media (1 ml) was mixed with 2 ml complete media, and H9C2 cells were treated with this mixture for 24 h. Cells were then fed with fresh DMEM complete media. On the second day, cells were incubated with media containing 1 μg/ml puromycin to select cells with successful incorporation of the frataxin gene.

Construction of frataxin-knockdown H9C2 cells. Frataxin knockdown cells were constructed using a lentiviral system containing short hairpin (sh)RNA for frataxin. The shRNA lentiviral backbone plKO.1 plasmid was purchased from Addgene [plasmid no. 10878, developed by and a kind gift of David Root’s laboratory (23)]. shRNA oligos were constructed according to the protocol described in the Addgene website (http://www.addgene.org/tools/protocols/plk0/). The constructed shRNA oligos and plKO.1 backbone were treated with AgeI and EcoRI (New England Biolabs) restriction enzymes and ligated using a Quick DNA ligase kit (New England Biolabs). The ligated vector was transformed into competent bacteria, and the plasmids were isolated using a Promega pure yield plasmid midi prep system. The purified virus was transfected to HEK-293T cells with the packaging plasmids mentioned above. Virus-containing media was collected on the third day after transfection and purified by passage through a 0.45-μm syringe filter. H9C2 cardiomyocytes were infected with the lentivirus, and cells that had incorporated the lentiviral gene were purified by 1 μg/ml puromycin treatment.

Immunoblots. Protein lysates from heart tissue and H9C2 cardiomyocytes were denatured in Lamelli buffer with DTT and heated at 95°C for 5 min. Proteins were resolved using standard Western blot analysis techniques and immunoblotted using anti-frataxin (1:750, Santa Cruz Biotechnology), HIF-1α (1:250, Santa Cruz Biotechnology), and α-tubulin (1:2,000, Developmental Studies Hybridoma Center, University of Iowa) primary antibodies and horseradish peroxidase-conjugated secondary antibody (1:2,000, Rockland). Blots were visualized after incubation with chemiluminescence reagent (Millipore) and imaged using a Bio-Rad gel dock system. Densitometry values were obtained using ImageJ software.

Promoter activity assay. PGL3 vectors, which constitute 1, 1.3, 2.0, and 2.5 kb upstream of the human frataxin promoter, were purchased from Addgene [plasmid nos. 14978, 14979, 14981, and 14980, developed by and a kind gift of Michael Ristow (35)]. H9C2 cells were transfected with these reporter vectors using Jetprime transfecting reagent (Polyplus). Cultures were cotransfected with a plasmid that encodes for a constitutively active HIF-1α mutant protein (plasmid no. 52636, Addgene, developed by and a kind gift of Eric
Huang’s laboratory (14) and pSV-β-galactosidase vector (Promega). After 36 h posttransfection, cells were harvested, and luciferase activity was measured using a luciferase assay system (Promega). Relative light units were standardized to β-Tubulin/H9251. Huang’s laboratory (14) and pSV-galactosidase vector (Promega). After 36 h posttransfection, cells were harvested, and luciferase -galactosidase vector (Promega). 

Fig. 1. Absence of hypoxia-inducible factor (HIF-1α) expression in the heart leads to extensive cardiac damage in response to ischemia-reperfusion (I/R) injury. A: Western blot analysis verifying that HIF-1α expression was absent in the heart (H) but not adipose tissue (Adp) or skeletal muscle (Skm) in cardiac-specific HIF-1α knockout (KO) mice. Furthermore, in loxP littermate controls, the presence of HIF-1α was observed in the heart. B: Genotype analysis verifying the presence of HIF-1α loxP and Cre recombinase expression in hearts from homozygous HIF-1α KO mice. loxP represents HIF-1α loxP, and Cre represents Cre recombinase, which is driven by the α-myosin heavy chain promoter. The top portion of the gel represents HIF-1α loxP identification, and the higher band represents the homozygous loxP genotype. Cre recombinase mice are shown in the bottom portion of the gel. Furthermore, the lower band indicates the internal control, and the top band represents the presence of the Cre recombinase gene. C: Analysis of the Evans blue and triphenyltetrazolium (TTC) stain. Damage to the heart after I/R injury (30 min of ischemia followed by 2 h of reperfusion) was determined by measuring the area at risk (AAR) from sham-operated (sham) and I/R hearts. The graphical analysis represents average values obtained from all animals in the same treatment group. D: The percent infarct represents area that was damaged (white) compared with the AAR (red). The graphical analysis is presented as a comparison with loxP sham treatment. E: Representative images of hearts from the I/R injury experiment. Evans blue and TTC staining indicate the ischemic region and perfused region in the hearts of mice subjected to I/R injury; blue represents the perfused region, red is the AAR, and white is the infarcted region. In response to I/R injury, extensive damage was observed in HIF-1α KO mouse hearts relative to the sham control, loxP, and Cre mice. All values in the graphically represented figures are means ± SE; n = 6 mice/group. ns, No significance. *P < 0.05.
Mitochondrial iron assays. Mitochondria were isolated as per the above-described protocol from ischemic and perfused heart tissues as well as cultured H9C2 cardiomyocytes for the ferroxine colorimetric assay (34). Previously in neuronal cell cultures, ferroxine assays were used to detect and quantify cellular iron accumulation. The ferroxine-iron complex that formed was measured by reading the absorbance at 550 nm in a multispectral plate reader (Bio-Tek Instruments).

Mitochondrial ROS assays. Mitochondrial ROS were measured using mitochondria-specific dihydrorhodamine indicator (Biotium). Dihydrorhodamine is an uncharged nonfluorescent ROS indicator that accumulates in the mitochondria and becomes oxidized to cationic rhodamine-123, which exhibits a green fluorescence. H9C2 cells and stable cell lines were stained according to the manufacturer’s protocol. Fluorescence was measured using a multispectral fluorescent plate reader (Bio-Tek) at excitation/emission wavelengths at 505/534 nm and imaged using a Nikon-TiS inverted confocal microscope.

Mitochondrial membrane potential assay. Mitochondrial membrane potential ($\Delta \Psi_m$) was measured by using tetramethylrhodamine ethyl ester (TMRE; Biotium). Cells were stained according to the manufacturer’s protocol, and signals were detected under a multispectral fluorescent plate reader (BioTek) at excitation/emission wavelengths at 548/575 nm.

MTT assay. Cell viability was assessed using the MTT cell viability assay kit (Biotium), and the assay was conducted according to the manufacturer’s protocol. Absorbance was measured at 570 nm using Bio-Tek plate reader. Values were standardized to a sulforhodamine B colorimetric assay.

Statistical analysis. I/R surgeries were performed with the investigator blinded to the treatment and genotype of the animal. Data are expressed as means ± SE. Results were normalized to the basal values and analyzed by ANOVA with Tukey’s post hoc analysis for significance between groups and within groups. Furthermore, a Student’s...
t-test (two tailed) was also performed to evaluate significance between groups. A minimum of 6 mice/group was used to achieve statistical significance. The level of significance was set at \( P < 0.05 \). All statistical calculations were performed on a computer using GraphPad Prism 5.0 software. All cell culture experiments were based on a minimum of five independent experiments.

RESULTS

Cardiac-specific HIF-1α KO mice display significant damage in response to I/R and have attenuated frataxin levels. HIF-1α is known as the master transcription factor that regulates the cellular response to hypoxia (40). It is known to induce protective mechanisms against I/R (28). However, the protective mechanism exerted by HIF-1α against ischemic injury upon mitochondria in cardiomyocytes remains relatively unknown. To better understand this, we developed a cardiac-specific HIF-1α KO mouse model, as described above and validated by Western blot analysis and PCR (Fig. 1, A and B). The Western blot analysis validated the absence of HIF-1α expression in heart tissue extracted from cardiac-specific HIF-1α KO mice. However, in these mice, HIF-1α protein was found to be expressed in adipose tissue and skeletal muscle as well as in hearts from control loxP mice (Fig. 1A). Also, the genotype analysis further confirmed the presence of the loxP site (Fig. 1B, top) and Cre gene (Fig. 1B, bottom) in homozygous HIF-1α KO mice. When subjected to 30 min of ischemia followed by 2 h of reperfusion, HIF-1α KO mice displayed significantly higher levels of damage (infarct size-to-area at risk ratio) than sibling loxP or Cre mice (Fig. 1, C–E). Although previous studies have verified that HIF-1α regulates factors associated with mitigating damage from I/R, few studies have validated HIF-1α-mediated signaling in the mitochondria.

To further investigate this, we analyzed protein lysates extracted from ischemic and perfused loxP and Cre mouse hearts and observed elevated levels of frataxin in ischemic regions compared with perfused regions of the same mice. Interestingly, HIF-1α KO mice had significantly attenuated frataxin expression in both ischemic and perfused tissues after exposure to I/R injury compared with loxP and Cre mice (Fig. 2, A and B). To further validate these observations, we treated a group of HIF-1α KO and loxP mice with DMOG. This compound inhibits prolyl hydroxylases, which target HIF-1α for ubiquitination and therefore stabilize HIF-1α under normoxic conditions. DMOG treatment significantly elevated HIF-1α expression as well as frataxin expression in the hearts of control loxP mice. However, we also observed a slight increase in HIF-1α and frataxin protein levels in the hearts of

![Western Blot for HIF-1α and Frataxin Proteins](image)}
cardiac-specific HIF-1α KO mice after DMOG treatment (Fig. 2, C–E). This may be due to the presence of other cell types, such as fibroblasts and endothelial cells, in the heart that express HIF-1α and increase frataxin expression in response to hypoxia induced by DMOG.

Increased expression of frataxin in response to ischemia in the hearts and hypoxia in H9C2 cardiomyocytes is mediated by HIF-1α. We conducted several experiments to identify whether HIF-1α can transcriptionally upregulate frataxin expression in cardiomyocytes. We first observed HIF-1α nuclear localization under hypoxia (1% O2 for 18 h) by immunolocalization (yellow punctate bodies) in H9C2 cells (Fig. 3A). To further validate if HIF-1α is involved in elevating frataxin levels, we used a RNAi system to knockdown HIF-1α in H9C2 cells. We demonstrated that HIF-1α RNAi can effectively attenuate HIF-1α nuclear localization in response to hypoxia in H9C2 cells (Fig. 3A). Furthermore, constitutively active HIF-1α mutant protein (P402A/P564A HIF-1α), which lacks the proline sites that are targeted for proteasomal degradation by HIF-1 prolyl hydroxylases, was also found to localize in the nucleus under normoxic conditions (Fig. 3A). As expected, our in vitro experiments revealed a significant increase in frataxin expression in H9C2 cardiomyocytes subjected to hypoxia or DMOG treatment (Fig. 3, B and C). However, when HIF-1α expression was attenuated using HIF-1α RNAi, a significant reduction was observed in frataxin expression in response to hypoxia as well as DMOG treatment in H9C2 cells (Fig. 3, B and C). These data suggest that HIF-1α acts as a key regulator for frataxin gene expression under hypoxic conditions.

To assess how HIF-1α induced an increase in frataxin expression, we conducted promoter activity assays using various reporter vectors containing one of four different regions of the human frataxin promoter upstream of the initiation site (1.0, 1.3, 2.0, and 2.5 kb) in H9C2 cells. We cotransfected these reporter vectors with the P402A/P564A HIF-1α vector and observed a greater than threefold increase in reporter gene activity for the 2.5-kb FXN promoter/luciferase constructs compared with normoxic cells containing the same constructs (Fig. 4A). These data validate that the presence of HIF-1α can induce frataxin promoter activity.

However, it has been previously reported that HIF-2α rather than HIF-1α preferentially binds to the mouse frataxin promoter in the mouse liver (26). To further validate the activity of HIF-2α on the human frataxin promoter, we conducted a promoter activity assay using the 2.5-kb frataxin promoter/luciferase reporter vector cotransfected with a vector that produces constitutively active human HIF-2α (P405A/P531A) protein. Unlike constitutively active HIF-1α, constitutively active HIF-2α was ineffective in stimulating the frataxin promoter in the same regions (2.5 kb) as measured by HIF-1α (shown in Fig. 4A). Furthermore, we could not observe a significant promoter response with constitutively active HIF-2α in the presence of RNAi for HIF-1α in H9C2 cells subjected to hypoxia or DMOG treatment (Fig. 4B). Therefore, the results from our previous in vivo, in vitro, and reporter analysis experiments indicated the presence of potential hypoxia response elements (HREs) for HIF-1α in the frataxin promoter.

To further understand the significance of HIF-1α regulation of frataxin protein expression, we completed a ChIP assay on loxP mice and HIF-1α KO mice subjected to either 30 min of ischemia or DMOG treatment. Our ChIP assays probed for six different regions in the frataxin promoter, which spanned the putative HRE regions that were predicted by our in silico experiments. Of the six regions we assessed, the ChIP assay revealed the presence of...
a functional HRE for HIF-1α between the 24260478 and 2426078 coordinates in the mouse frataxin promoter (Fig. 5A). A schematic diagram of the putative HRE region for HIF-1α in the mouse frataxin promoter that showed a positive response to our ChIP assay is shown in Fig. 5B. Furthermore, the sequences of the primers that probed for six regions in the mouse frataxin promoter in the ChIP assay are shown in Fig. 5C.

Increased frataxin expression under hypoxia attenuates iron accumulation in mitochondria in cardiomyocytes. Frataxin protein is known to regulate iron homeostasis in the mitochondria in yeast cells (3, 12). Therefore, we hypothesized that increased frataxin levels under hypoxic conditions may play a role in regulating mitochondrial iron homeostasis in cardiomyocytes. To further assess this hypothesis, we constructed a FXN-OE H9C2 cell line and frataxin knockdown cell lines using lentiviral systems. These stable cell lines were characterized by Western blot analysis, in which the FXN-OE cell line displayed a more than twofold increase in frataxin expression compared with control cells (Fig. 6A). Furthermore, two frataxin knockdown cells lines, KD-2 and KD-3, had ~40% and 80% reductions in frataxin expression, respectively (Fig. 6, A and B). The levels of frataxin significantly increased in all the cell lines under hypoxic stress (Fig. 6, A and B). The 21-mer target sequences that were designed to generate the shRNA to create the frataxin knockdown cell lines are shown in Fig. 6C. Strand 1 failed to significantly suppress frataxin expression in H9C2 cells. Strands 2 and 3 were used to construct the KD-2 and KD-3 cell lines, respectively.

**Fig. 5.** HIF-1α interacts with the frataxin promoter [chromatin immunoprecipitation (ChIP) assay]. A: validation of the presence of functional HRE region in the frataxin promoter was accomplished by a ChIP assay that compared ventricular lysate from loxP and HIF-1α KO mice exposed to treatments including sham or ischemia for 30 min or DMOG (1 mg) for 2 h. Each experiment was repeated with three different mice per group, yielding similar results. The image shown here best represents the interaction of HIF-1α with a putative HRE in the frataxin promoter. B: map showing the HRE region in the frataxin promoter. This is the same site to be recognized by HIF-1α in response to ischemia or chemical hypoxia (DMOG) shown in A. C: sequences of the primers used to probe for six different regions in the mouse frataxin promoter in the ChIP assay. Primer set 5, shown in the table, is shown in B. Primer sets 1–4 represent regions 1–2,000 bp upstream of the initiation site containing the potential HRE for HIF-1 in the frataxin promoter. Primer set 6 represents a region upstream of the 2.5-kb region of the frataxin promoter.

<table>
<thead>
<tr>
<th>Primer #</th>
<th>Primer sequence for ChIP assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Forward AGCCCTTCAATTCAGCCTGC</td>
</tr>
<tr>
<td>2</td>
<td>Forward TGCTGCTGGGGTACAGGACT</td>
</tr>
<tr>
<td>3</td>
<td>Forward CGCCCTGACCTTACAGCCTTCA</td>
</tr>
<tr>
<td>4</td>
<td>Forward ATGGCCACACTTGGTACACTCA</td>
</tr>
<tr>
<td>5</td>
<td>Forward AGCCCTTCAATTCAGCCTGC</td>
</tr>
<tr>
<td>6</td>
<td>Forward TGCTGCTGGGGTACAGGACT</td>
</tr>
</tbody>
</table>
HIF-1α-FRATAxin SIGNALING IN THE ISCHEMIC HEART

Fig. 6. Hypoxia stimulates the increase in frataxin expression. A: representative blot of four independent experiments demonstrating changes in frataxin levels in response to hypoxia. Changes in frataxin protein levels were determined by Western blot analysis in H9C2 control (Con) cells, frataxin overexpressing (FXN-OE) H9C2 cells, and frataxin knockdown (KD-2 and KD-3) under normoxic (20% O2) and hypoxic (1% O2) stimulation for 18 h. B: bar graph representing average densitometric values obtained for frataxin expression from H9C2, FXN-OE, KD-2, and KD-3 cells exposed to normoxic (20% O2) and hypoxic (1% O2) for 18 h. The graphical analysis reflects averages from four independent experiments. Absolute densitometric values were standardized to α-tubulin and then normalized to normoxic H9C2 control cells. Values are presented as means ± SE and were analyzed by ANOVA with Tukey’s post hoc analysis for significances between groups and within groups. *p < 0.05; **p < 0.01; ***p < 0.001. C: strands representing the sequences of the oligos constructed to develop the lentiviral vectors for frataxin knockdown cell production. Strands 2 and 3 were used to develop KD-2 and KD-3 frataxin knockdown cells, respectively. Strand 1 failed to attenuate the frataxin protein expression significantly (data not shown).

To better understand the effects of HIF-1α-frataxin signaling in mitochondrial iron homeostasis, we conducted ferrozine assays to evaluate iron levels in isolated mitochondria from mice hearts and from H9C2 cell lines. Interestingly, we observed that the ischemic regions of HIF-1α KO mice displayed the highest levels of iron in the mitochondria among all the groups (Fig. 7A). To better understand the cardioprotective role of HIF-1α-frataxin signaling activation against I/R injury, we treated mice with DMOG and observed an increase in mitochondrial iron levels in HIF-1α KO mice treated with DMOG relative to control loxP mice (Fig. 7B).

Among the two frataxin knockdown cell lines, the KD-3 cell line had markedly elevated mitochondrial iron levels relative to control cells under normoxic conditions, which further increased under hypoxic conditions (Fig. 7C). Additionally, treatment of KD-2 and KD-3 cells with the HIF-1 inhibitor further increased the mitochondrial iron accumulation under normoxia. Interestingly, there were minimal changes in the mitochondrial iron content observed between normoxic and hypoxic H9C2 cells. However, HIF-1 inhibition under hypoxic conditions significantly elevated mitochondrial iron levels in H9C2 cardiomyocytes. Most significantly, HIF-1α inhibition failed to increase iron levels in the FXN-OE cell lines exposed to hypoxia. Finally, mitochondrial iron levels were markedly high in hypoxic KD-3 cell lines, and these levels were further elevated in response to the HIF inhibitor (Fig. 7D). These data suggest that HIF-1α can mitigate mitochondrial iron accumulation in response to hypoxic stress in H9C2 cardiomyocytes. Furthermore, frataxin is centrally involved in this process.

Frataxin-mediated iron buffering helps to attenuate ROS formation and maintain mitochondrial membrane potential under hypoxia. Previous publications have confirmed that doxorubicin, a cancer chemotherapeutic agent, induced mitochondrial iron accumulation resulting in ROS production and oxidative stress in cardiomyocytes (1). Therefore, this study suggested that maintaining iron homeostasis in mitochondria is vital for cell survival. Based on our observations, frataxin plays a significant role in mitochondrial iron homeostasis under hypoxic insult, resulting in the prevention of ROS production and the subsequent oxidative stress.

We observed that H9C2 cells treated with HIF-1 inhibitor induced the highest ROS production under hypoxia (Fig. 8, A and B). However, this effect was prevented in FXN-OE cells (Fig. 8, C and D) as well as in H9C2 cells treated with both HIF-1 inhibitor and the mitochondrial iron chelator pyridoxal isonicotinoyl hydrazine (PIH; Fig. 8A). The ability of PIH to effectively regulate mitochondrial iron levels in surplus conditions has been previously established (33). Furthermore, ROS production was insignificant in FXN-OE cell lines treated with HIF-1 inhibitor under hypoxia (Fig. 8D). A previously published study (6) has linked increased ROS production to severe mitochondrial injury, including reduced mitochondrial ΔΨm (6). Maintenance of ΔΨm is vital for cells that have high energy requirements, specifically cardiomyocytes, and attenuation of ΔΨm induces injury to cardiomyocytes (38). We assessed ΔΨm in cells using the fluorescent probe TMRE. H9C2 cells that were treated with HIF-1 inhibitor and exposed to hypoxia had the lowest ΔΨm compared with the other treatment groups. These data were comparable

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00875.2014 • www.ajpheart.org

Design of shRNA for frataxin knock down

<table>
<thead>
<tr>
<th>Target sequence sense</th>
<th>Hair pin</th>
<th>Target sequence antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strand 1: 5’ AATTCTTTGAGACCTGTGAC 3’</td>
<td>TTAGAAGACTCTGGAAGCTGAGCTC</td>
<td>GAGCTCAGAGTTCCAGAAGTCTTCAATTAA</td>
</tr>
<tr>
<td>Strand 2: 5’ AAGAACTGGGTGTACCTTCAAGAGCTAGCTGACCACGAGGTA</td>
<td>TGCCGAATCACCCGAGGTTTTCTCTGAGACCACCATGAGGTA</td>
<td>ATGGGAGTACACCCAGTTTGGTGTT</td>
</tr>
<tr>
<td>Strand 3: AACACAAAGACTGACGGCACTTCGTTCTCTGAGACCACCATGAGGTA</td>
<td>AAGAGTGCTACCCGAGGTTTTCTCTGAGACCACCATGAGGTA</td>
<td>GTTGAGTACACCCAGTTTGGTGTT</td>
</tr>
</tbody>
</table>
existing studies have demonstrated various protective mechanisms offered by HIF-1α activation against I/R injury in the heart. In the present study, we provide novel evidence for the cardioprotective mechanisms triggered by HIF-1α against I/R injury, by regulating the mitochondrial protein frataxin. We further demonstrated that HIF-1α-mediated elevation of frataxin levels conferred cardiac protection by maintaining \( \Delta \Psi_m \), thereby preserving mitochondrial integrity in cardiomyocytes under oxidative stress.

Our in vivo and in vitro models demonstrated that frataxin expression is increased in response to HIF-1α activation. Furthermore, attenuated frataxin expression in HIF-1α KO mice subjected to I/R injury as well as decreased frataxin expression in the presence of HIF-1 RNAi in hypoxic H9C2 cell culture further consolidated the direct effects of HIF-1α activation upon frataxin expression. Our reporter activity assays confirmed the direct effect of HIF-1α on the human frataxin promoter. Moreover, we validated the presence of a functional HRE region in the murine frataxin promoter between 24260478 to 24260678 coordinates. However, previous work by Oktay et al. (26) demonstrated that HIF-2α rather than HIF-1α is responsible for governing frataxin levels in hepatocellular carcinoma (HCC) cells.
Fig. 8. Inhibition of HIF-1α induces an increase in mitochondrial ROS formation under hypoxic stress. A: representative images showing inhibition of HIF-1α by HIF-1 inhibitor in hypoxic H9C2 cells (1% O₂, 18 h) increased mitochondrial ROS formation, as measured by using mitochondrial specific dihydrorhodamine dye and imaged by fluorescence microscopy. This effect was attenuated with treatment with the mitochondria-specific iron chelator pyridoxal isonicotinoyl hydrazine (PIH). B: graphical analysis showing averages of ROS measurements standardized to total protein concentrations. Values are presented as means ± SE of four independent experiments normalized to control normoxic H9C2 cells and analyzed by ANOVA with Tukey’s post hoc analysis for significances between groups and within groups. C: the FXN-OE cell line displayed protection against ROS formation when HIF-1 was inhibited and cells were exposed to hypoxic stress. D: graphical analysis showing averages of ROS measurements standardized to total protein concentrations. Values are presented as means ± SE of four independent experiments normalized to control normoxic H9C2 cells. Statistical analysis was determined by ANOVA with Tukey’s post hoc analysis for significances between groups and within groups. Images were photographed using a Nikon inverted TS fluorescence microscope. ***p < 0.001.
cytes in mice. HIF-1α and HIF-2α have structural similarities except in their transactivation domains and are controlled in a similar fashion under normoxic conditions. The genes regulated by HIF-1α and HIF-2α overlap; however, several reports have implicated differential gene regulation and differential activation of these two transcription factors under stress (21, 25). Nonetheless, our reporter activity assays demonstrated that HIF-1α exerted a profound effect on the human frataxin promoter, whereas HIF-2α failed to produce a response. Furthermore, we observed a significant reduction of frataxin expression in the hearts of cardiac-specific HIF-1α KO mice subjected to I/R injury, indicating the profound effect of HIF-1α in the murine heart. Our animal model is specific for HIF-1α and allowed us to critically evaluate HIF-1α activity on the frataxin promoter in the ischemic heart. These findings are in line with previous findings that used shRNA against HIF-1α to suppress frataxin expression under hypoxic stress in human glioblastoma cells (9). However, compared with the work by Oktay et al. (26), the data provided in the present study do not explain the exact cause for the differential response seen in the frataxin promoter in response to HIF-1α and HIF-2α. The differential responses observed in these studies may be attributed to species-specific or organ-specific responses but require additional experiments to better understand the exact roles of HIF-1α and HIF-2α in hypoxia in the heart. Furthermore, as discussed above, our findings are in contrast to Oktay et al.’s findings. To better explain these findings, we compared the promoters from rodents (mouse and rat) to the human promoter and observed that the upstream regulatory regions of frataxin are poorly conserved among mammals. Therefore, we found it difficult to interpret whether HIF-1α regulation of human frataxin gene expression can be extrapolated to rodents.

Although the exact function of frataxin is not fully elucidated and remains controversial, it is well accepted that frataxin is an important mediator of mitochondrial iron homeostasis by acting as a chaperone or a storage protein for iron. This enables the maintenance of iron in a nontoxic bioavailable form (29). Additionally, loss of frataxin in eukaryotic cells leads to mitochondrial iron accumulation and the subsequent production of highly toxic free radicals (7). This conclusion was further confirmed by our data, which clearly demonstrated that ablation of frataxin leads to increased mitochondrial iron accumulation in cardiomyocytes under normoxic and hypoxic stress. Furthermore, overexpression of frataxin reversed this effect.

The mechanism by which hypoxic stress leads to iron accumulation in the mitochondria is not fully understood. However, based on previous publications, it can be postulated that the accumulation of free iron in the mitochondria may occur during the switch from oxidative phosphorylation to glycolysis under hypoxia. Under hypoxic stress, activation of HIF-1α shifts the energy regulatory axis away from oxidative phosphorylation toward glycolysis (17, 19). This conversion is protective as continuation of oxidative phosphorylation under hypoxic conditions instigates the production of toxic free radicals due to the absence of oxygen, which normally accepts the electrons from the electron transport chain in the mitochondria (15).

However, unregulated iron overload in cardiomyocytes induces mitochondrial ROS production and promotes mitochondrial damage by triggering opening of the mitochondrial per-
meability transition pore (MPTP). The MPTP is a nonspecific pore located in the inner membrane of mitochondria and remains relatively closed under normal physiological conditions. Moreover, it offers permeability to only selective metabolites and ions and thus help to maintain $\Delta \Psi_m$ in the mitochondria. Maintenance of proper $\Delta \Psi_m$ is crucial for cells that have high energy requirements, such as cardiomyocytes. Dissipation of $\Delta \Psi_m$ through MPTP opening under pathological conditions releases proapoptotic factors and Ca$^{2+}$ stored in the mitochondria, leading to cardiomyocyte death and extensive tissue damage (38). In addition, a previous study (10) has demonstrated that expression of frataxin in hypoxic-ischemic cardiomyocytes, protection against I/R injury (41, 44). Furthermore, HIF-1α-frataxin expression in hypoxic/ischemic cardiomyocytes, and thus the reduced ROS production under oxidative stress seen in our experiments, may also contribute to the protection observed in these hearts when subjected to I/R injury. Therefore, we suggest that induction of the HIF-1α-frataxin signaling pathway during the ischemic phase may protect against iron overload, and, therefore, this protection may extend toward the reperfusion phase by mitigating MPTP opening.

However, the possibility of the presence of cardioprotective mechanisms mediated by frataxin other than those described above cannot be disregarded. In a FRDA mouse model, increased oxidative stress was observed in dorsal root ganglia due to a frataxin-dependent defect in the transcription of nuclear factor erythroid 2-related factor 2, which exerts antioxidant effects in biological systems (36). Furthermore, the ability of frataxin to exert antioxidant effects by activation of glutathione peroxidase has also been previously documented (37). Therefore, we acknowledge that the antioxidant effects of frataxin independent of iron regulation may also contribute to the reduced ROS production under oxidative stress seen in our experimental models. Furthermore, alternative cardioprotective mechanisms conferred by HIF-1α signaling against I/R injury cannot be disregarded. It has been recently found that autophagy may be a highly valuable mechanism for cardioprotection against I/R injury (41, 44). Furthermore, HIF-1α regulates autophagy in response to ischemia (22).

In summary, we found that suppression of HIF-1α attenuates frataxin expression in hypoxic/ischemic cardiomyocytes, which leads to increased mitochondrial free iron content and subsequent ROS production. Moreover, we observed that overexpression of frataxin attenuates the mitochondrial iron accumulation stimulated by hypoxia, especially under HIF-1α deficiency. This, in turn, mitigates ROS production and MPTP opening, thus enabling cardiomyocytes to sustain viability. Therefore, we suggest that activation of the HIF-1α-frataxin signaling pathway offers protection to cardiomyocytes by preventing iron accumulation, thus preserving mitochondrial integrity under hypoxic stress. We postulate that frataxin may exert its beneficial effects by acting as an iron storage protein in hypoxic cardiomyocytes and subsequently facilitates the maintenance of $\Delta \Psi_m$ and promotes cell survival.

One of the limitations in the present study is that it does not validate the state of iron in the mitochondria under hypoxia/ischemia and how frataxin modulates this free iron. Also, we acknowledge the absence of physiological cardiac performance evaluation in response to I/R injury in our in vivo models. A significant part of our future work will address these issues to further consolidate the significance of HIF-1α-frataxin signaling in the heart.

GRANTS

This work was supported by an internal grant from the Auburn University Research Initiative in Cancer Research as well as a generous startup package to R. Amin from Auburn University.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES