Transcriptional Regulation of SDHa Flavoprotein by Nuclear Respiratory Factor-1 Prevents Pseudo-hypoxia in Aerobic Cardiac Cells

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Nuclear respiratory factor-1 (NRF-1) is integral to the transcriptional regulation of mitochondrial biogenesis, but its control over various respiratory genes overlaps other regulatory elements including those involved in O2 sensing. Aerobic metabolism generally suppresses hypoxia-sensitive genes, e.g. via hypoxia-inducible factor-1 (HIF-1), but mutations in Complex II-succinate dehydrogenase (SDH), a tumor suppressor, stabilize HIF-1, producing pseudo-hypoxia. In aerobic cardiomyocytes, which rely on oxidative phosphorylation, we tested the hypothesis that NRF-1 regulates Complex II expression and opposes hypoxia-inducible factor-1. NRF-1 gene silencing blocked aerobic succinate oxidation, increasing nuclear HIF-1α protein prior to the loss of Complex I function. We postulated that NRF-1 suppression either specifically decreases the expression of one or more SDH subunits and increases succinate availability to regulate HIF-1 prolyl hydroxylases, or stimulates mitochondrial reactive oxygen production, which interferes with HIF-1α degradation. Using promoter analysis, gene silencing, and chromatin immunoprecipitation, NRF-1 was found to bind to the gene promoters of two of four nuclear-encoded Complex II subunits: SDHa and SDHd, but the enzyme activity was dynamically regulated through the catalytic SDHa flavoprotein. Complex II was inactivated by SDHa silencing, which led to aerobic HIF-1α stabilization, nuclear translocation, and enhanced expression of glucose transporters and heme oxygenase-1. This was unrelated to mitochondrial ROS production, reversible by high α-ketoglutarate concentrations, and coherent with regulation of HIF-1 by succinate reported in tumor cells. These findings disclose a novel role for NRF-1 in the transcriptional control of Complex II and prevention of pseudo-hypoxic gene expression in aerobic cardiac cells.

Transcriptional regulation of mitochondrial electron transport chain (ETC)2 proteins requires the expression of both nuclear- and mitochondrial-encoded genes that are specifically coordinated by a small number of nuclear factors (e.g. NRF-1, NRF-2, and ERRα) and co-activators of the PGC-1 family (e.g. PGC-1α and PRC) (1–3). However, these nuclear factors vary in their degree of control of various mitochondrial oxidative pathways (4, 5). The NRF-1 transcription factor binds as a homodimer to promoter sites in nuclear-encoded genes involved in the regulation of mitochondrial biogenesis as well as genes encoding selected subunits of the five respiratory complexes (6). NRF-1 also binds regulatory elements in genes involved in cell cycle control and proliferation, overlapping most notably with E2F target genes involved in DNA replication, mitosis, and cytokinesis (7). NRF-1 occupancy has been reported in 5% of gene promoters in living human cells (7), and it is strongly up-regulated by inflammation and mtDNA damage (8). Although NRF-1 has been implicated in nuclear activation of genes encoding subunits of all five respiratory complexes (1), its role in the integrative expression of different mitochondrial electron transport chain proteins is neither clearly elucidated nor is it known whether it has a role in oxygen (O2) sensing.

Mammalian cells sense O2 deprivation by activating the transcription of adaptive hypoxia-inducible genes: glucose transporters, vascular endothelial growth factor, and erythropoietin (9–11). This response facilitates glycolytic ATP production until O2 delivery improves via enhanced erythropoiesis and angiogenesis. O2 sensing is regulated in part by hypoxia-inducible factor-1 (HIF-1), a heterodimer of HIF-1α and HIF-1β of the ARNT family of transcription factors (10). Both subunits are required for DNA binding and gene activation, but only HIF-1α is regulated by the cellular O2 tension. In the presence of O2, the HIF-1α subunit is targeted for proteosomal degradation by prolyl hydroxylases (PHD 1–3) that utilize α-ketoglutarate in the presence of ferrous iron and ascorbate to hydroxylate specific proline residues catalytically and liberate CO2 and succinate (11).

The stabilization of HIF-1α under aerobic conditions, called pseudo-hypoxia, is a feature of some tumors related to inactivation of mitochondrial Complex II, succinate dehydrogenase

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2 The abbreviations used are: ETC, electron transport chain; HIF-1, hypoxia-inducible factor-1; NRF, nuclear respiratory factor; PHD, prolyl hydroxylase; ROS, reactive oxygen species; SDH, succinate dehydrogenase; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; nt, nucletide; ChIP, chromatin immunoprecipitation assay; scRNA, scrambled RNA; si, small interfering; DMOG, dimethylxalylglycine; DCM, dichloromethane; DCM/CO, CO-generating dichloromethane; α-KG, α-ketoglutarate; RT, reverse transcriptase.
**NRF-1 and SDHa Regulation**

(SDH; succinate:ubiquinone oxidoreductase) (12–14), highlighting its role as a mitochondrial tumor suppressor. HIF-1 activation by SDH inactivation has been proposed to involve one of two mitochondrial mechanisms, one entails mitochondrial reactive oxygen species (ROS) generation, while the other requires egress of mitochondrial succinate; either would inhibit cytoplasmic HIF PHDs and stabilize the α-subunit (12, 15). In hypoxia, the connections between the mitochondrial ETC, ROS production, and HIF-1α expression are physiologically important, and the mechanisms have generated some controversy (15–18).

Understanding ETC involvement in hypoxic gene activation is also necessary to comprehend respiratory chain anomalies involved in disease pathogenesis, including human cardiomyopathy (19, 20). Complex II inhibition or the generation of ROS at Complex III leading to HIF-1 activation have implicated ETC disturbances to hypoxic gene expression; moreover, Complex I or IV defects that allow the molecular O₂ concentration in the cell to rise may destabilize HIF-1α, e.g. as shown by nitric oxide binding to the oxidase (21). Under such conditions, down-regulation of NRF-1-regulated gene expression could increase HIF-1α stability by multiple mechanisms. Here we tested the hypothesis that NRF-1 interference promotes HIF-1 activity in aerobic cardiomyocytes by either: (i) decreasing specific SDH subunit expression and increasing succinate availability, thereby regulating prolyl hydroxylase activity, or (ii) enhancing mitochondrial reactive oxygen species (ROS) production at Complex II or III that prevents the degradation of the HIF-1 α-subunit.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Two cell lines were used: H9c2 cells derived from embryonic rat ventricle (American Type Culture Collection) and primary ventricular cardiomyocytes isolated from rat heart. H9c2 cells were seeded at 5 × 10⁵ cells/100-mm plate and cultured at 37 °C in a 5% CO₂ humidified atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (high serum, growth-promoting medium) renewed every second day. Cells were switched to DMEM supplemented with 1% fetal bovine serum (low serum, differentiation-promoting medium, DM) for differentiation after reaching subconfluence (70–80%, 3–4 days). H9c2 cell morphology was followed daily by phase contrast microscopy to confirm differentiation, including myotube formation and expression of myogenic transcription factors, calcium channel proteins, and LIM protein FHL2 (22, 23). Rat ventricular cardiomyocytes were isolated using a kit (Worthington, Lakewood, NJ) from 3-day-old Sprague-Dawley rats under an approved animal protocol and the cardiomyocytes cultured at 37 °C (density of 6 × 10⁴ cells/cm²) in DMEM/F12 supplemented with antibiotics, 10% horse serum (Sigma-Aldrich), 5% heat-inactivated fetal bovine serum (HyClone, Logan, UT), and 10 μg/ml fibronectin. These cardiomyocytes beat spontaneously, and their purity was >97% by immunocytochemical staining with anti-cardiac sarcomeric α-actinin and fluorescence microscopy.

Cells for transfection were grown in standard culture dishes, and those for immunofluorescence and fluorescent oxidative stress assays were grown on Nunc Lab-Tech Permanox glass chamber two-well slides (Fisher Scientific). All experiments were performed in separate triplicate cultures, and ten groups were analyzed: control cells grown in air + 5% CO₂ (aerobic conditions); cells grown in aerobic conditions plus dichloromethane (DCM, 50–100 μM for 24 h); aerobic cells exposed to DMOG (1 mM) for 24 h; aerobic cells fed α-ketoglutarate (2 mM); aerobic cells transfected with small interfering (si) SDHa, siNRF-1, or SDHα, or siNRF-1 plus SDHα or with scrambled siRNA; or aerobic cells incubated with 5 μM MG132 for 24 h. Final doses were chosen from preliminary studies.

**Antibodies and Other Reagents**—A specific polyclonal anti-NRF-1 was developed and characterized using a unique peptide of amino acid sequence 262–279 of human NRF-1, highly homologous to the mouse and rat (24, 25). The peptide was conjugated to keyhole limpet hemocyanin and injected into rabbits. Antiserum was screened for specificity against recombinant NRF-1 produced by in vitro translation in reticulocyte lysate (Promega, Madison, WI). The antibody was adsorbed to the peptide and tested by Western blot analysis to confirm NRF-1 specificity.

Anti-tubulin and anti-β-actin were obtained from Sigma, and anti-myogenin, Glut1, Glut2, and Glut4 antibodies from Santa Cruz Biotechnology. Anti-HIF1α was from either Transduction Laboratories (Lexington, KY) or Novus Biologicals (Littleton, CO). Anti-Hyp-P564 antibody was obtained from Genaxis. All other chemicals and reagents, including RPMI and DMEM with 25 mM Hepes and 4.5 g/liter glucose, were from Sigma.

**Gene Silencing**—These experiments were performed using small interfering NRF-1 (siNRF-1) or siSDHa duplexes to target sequences in the open-reading frames of NRF-1 or SDHα mRNA. Multiple nucleotide sense and antisense siRNAs were synthesized and obtained in annealed form from Ambion. SiRNA target sequences were submitted to BLAST searches against other rat genome sequences to ensure specificity. After preliminary studies, one pair of siRNA sequences was selected and transfected at concentrations of 40 or 80 μM into cells using FuGENE 6 (Roche Applied Sciences) sense NRF-1, 5′-GGAGGUAUAUUCAGAGCG-3′, and antisense NRF-1, 5′-CACAGUCUGAAAUUAACCCCA-3′ and sense SDHα, 5′-GCAAGAGGCAUCCCCAAtt-3′, and antisense SDHα, 5′-UAAGCGGAGUCCUUCUGCtc-3′. A scrambled negative control siRNA was also used. The experiments were conducted at transfection efficiencies of 60–80% determined by co-transfection with fluorescent double-stranded oligonucleotides. Inhibition of NRF-1 by siNRF-1 was established by Western blot analysis and inhibition of SDHα by siSDHα by RT-PCR using specific primers for rat SDHα and by Western blot with mouse monoclonal anti-human SDHα and SDHβ (Invitrogen). Cardiomyocytes were transfected with SDHa cloned in pCMV6-XL5 expression vector (OriGene, Rockville, MD) 48 h after plating using FuGENE 6 and transfection efficiency was checked by co-transfection with a green fluorescent protein construct. Cells were exposed to the transfection mix for 8 h,
then grown in PC-1/F-12 minimal essential medium for 24, 48, or 72 h.

**Nuclear and Mitochondrial Proteins and Respiration**—The nuclear and mitochondrial fractions were obtained from disrupted cells by differential centrifugation. Nuclear extracts were prepared in 0.3 M NaCl, 1 M urea, 1% Nonidet P-40, 25 mM HEPES (pH 7.9), and 1 mM dithiothreitol (26). Respiration was measured by polarography at 35 °C in digitonin (0.02%)-permeabilized cells in a standard respiration buffer with or without inhibitors 50 μM antimycin A, or substrates: 1 mM ascorbate + 100 μM TMPD, 5–10 mM succinate, or 2.5 mM glutamate + 2.5 mM malate (25, 27). Protein concentrations were determined by the Bradford method (Bio-Rad).

**Western Blot Analysis**—Cells were transfected with siNRF-1 or siSDHa or scrambled RNA (scRNA) using FuGENE 6 and grown for 24, 48, or 72 h post-transfection. For Western blots, cells were lysed in 50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% Triton, 0.1 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 150 mM mercaptoethanol. The samples were sonicated, boiled in Laemmli buffer (10–20 μg of protein), followed by electrophoresis on 8 or 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidifluoride membranes. Nuclear extracts were also prepared from cultured cells to check for HIF-1α nuclear protein (24). For electrophoresis, 15–20 μg of cellular, nuclear, or mitochondrial protein per lane were used, membranes blocked with 5% dry defatted milk, incubated with monoclonal anti-HIF-1α antibody or anti-NRF-1 or anti-SDH, followed by secondary antibody and detection using enhanced chemiluminescence (Amer sham Biosciences).

**Immunoprecipitation**—Cell lysates were immunoprecipitated using anti-HIF-1α antibody conjugated to protein A-Sepharose. The precipitates were washed in 80 mM Tris-HCl, pH 7.4, 200 mM NaCl, 20 mM EDTA, 50 mM NaF, 2 mM Na3VO4, 1% (v/v) Nonidet P-40, 50 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 10 mg/ml aprotinin and further washed with 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 100 mM Na3VO4. The samples were boiled in Laemmli buffer and separated on 8% SDS-PAGE, transferred to nitrocellulose, and blotted with Anti-Hyp-P564. The blots were developed, and spots quantified on digitized images from the mid-dynamic range. Analysis of band intensities was performed by Image-Quant (Bio-Rad).

**RNA Extraction and Quantitative Real-time RT-PCR**—Total RNA was extracted from cells followed by reverse transcription with a first-strand RT-PCR kit (Promega) using the manufacturer’s instructions. Preliminary assays were done by conventional RT-PCR using the primer sequences in Table 1. Quantitative real-time PCR was performed with TaqMan gene expression and premix assays for SDHa, -b, -c, and -d (Applied Biosystems) on an ABI Prism 7000 (Applied Biosystems) Real-time PCR system. To detect SDH subunit induction, relative expression for each assay was normalized against GAPDH (TaqMan® Endogenous Controls) using the recommended comparative threshold cycle CT method. Because of the exponential PCR reaction, a difference of n in Cn values represents a 2-fold difference in transcript levels. PCR was performed in triplicate, and experiments were repeated in triplicate.

**In-gel Enzyme Activity Staining**—In-gel staining of SDH activity relies on formazan precipitation as purple crystals derived from the reduction of nitro-blue tetrazolium (NBT). The colorless dye accepts hydrogen during oxidation by NADH and FADH2 dehydrogenases. NBT accepts hydrogen directly from NADH systems, e.g. Complex I, whereas dye release is delayed by an artificial electron carrier, phenazine methosulfate (PMS). In control H9c2 cells or those transfected with siSDHa for 24 h, the plasma membrane was disrupted, the nuclei removed, and the mitochondrial complexes separated by non-denaturing PAGE. The gels were rinsed in distilled H2O and incubated in PMS and NBT buffer for varying times in the dark at room temperature (28). The reactions were stopped by fixation in 45% (v/v) methanol and 10% (v/v) acetic acid and the gels de-stained overnight in the same solution.

**Chromatin Immunoprecipitation Assays**—H9c2 cells (~4.0 × 106) cultured in 15-cm plates were treated 100 μM DCM or transfected with siNRF-1. After 24 h, cells were treated with 1% formaldehyde (cross-linker) for 7.5 min, harvested, sonicated (into ~500 bp fragments) in media to achieve a final concentration of 1%, and incubated at 37 °C for 30 min. Formaldehyde was quenched with 0.125 M glycine. Cells were washed with PBS, harvested, and processed with the ChIP IT Assay Kit (Active Motif) and NRF-1 antibody. After ethanol precipitation, DNA was resuspended in 200 μl 10× binding buffer, and 2–5 μl used for the PCR template. Input samples representing 1% of total DNA were diluted at 1:5 and IP fractions 1:2. PCR was carried out on 1-μl samples using validated primer sets for SDHa, SDHb, SDHc, and SDHd 5′-untranslated regions (Table 1). Total input DNA was also tracked through the assay by PCR using primers that amplify 223 bp from the β-actin promoter. The primer mix came from Active Motif (Carlsbad, CA) and was used as recommended. Preimmune rabbit serum (IgG) was used for IP and purified DNA for PCR of the SDH subunits (Table 1). PCR products were analyzed on 2% ethidium bromide-stained agarose gels and normalized to total DNA in each sample.
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*Immunofluorescence Microscopy*—H9c2 cell were grown in two-well chamber slides to 60–70% confluence to avoid hypoxia. Cells were transfected with siSDHa for 24 h or transfected with siSDHb and incubated with 2 mM α-ketoglutarate for 6 h. Cells were treated with MitoTracker Green (1 μM) for 10 min at 37 °C, fixed in 4% paraformaldehyde, and then 1% Triton X-100 in PBS for 15 min at room temperature. Cells were incubated for 30 min at room temperature with 10% normal horse serum (Dako Corp.) in PBS for 24 h with anti-HIF-1α at 1:100 in PBS containing 5% horse serum. After three 15-min washes in PBS with gentle agitation, cells were labeled for 1 h with goat anti-mouse FluoroLink Cy3-labeled (Molecular Probes) at a 1:100 dilution in PBS containing 5% horse serum, washed three times in PBS, cover-slipped using Slow Fade (Invitrogen) mounting medium, and observed by confocal microscopy (Zeiss 410 LSCM). For all samples, images were collected at ×620 magnification in the fluorescence mode using identical settings, and images merged electronically.

*ROS Production*—To localize ROS production, cells were transfected or exposed to DCM/CO and incubated 24 h later with MitoSox (1 μM) and MitoTracker Green (Molecular Probes) for 10 min followed by fluorescence confocal microscopy at 568 nm (MitoSox) and 488 nm (MitoTracker) on a Model 410 LSCM (Carl Zeiss MicroImaging, Inc). Control and treated samples were scanned at ×620 using the same parameters, and the images collected in fluorescence mode and merged electronically.

*Statistics*—Grouped data were expressed as means ± S.E. Statistical analyses were performed by two-way analysis of variance using commercial software. A value of p < 0.05 was accepted as significant.

**RESULTS**

**Respiration and Mitochondrial Complex II Activity**—Oxidative phosphorylation substrate utilization in H9c2 cardiomyocytes was examined before and after NRF-1 silencing. As a positive control, NRF-1 expression, which in these cells involves mitochondrial oxidant production, was stimulated with DCM/CO (25). At the baseline, the cells expressed immunoreactive nuclear NRF-1 protein, which was increased 2-fold by DCM/CO and blocked (>90%) 24 h after transfection with siRNA (Fig. 1A). siRNA had no effect on NRF-1 protein expression.

Within 24 h after NRF-1 silencing, cell respiration became compromised; however, loss of FADH-linked respiration appeared early and was more sensitive to siNRF-1 than NADH-linked (malate + glutamate) respiration (Fig. 1B). siRNA had no effect on respiration, and the oxidation of ascorbate-TMPD (+antimycin A) was intact 24 h after NRF-1 silencing (data not shown). NRF-1-dependent gene expression is thus necessary for carbon entry into the ETC in rat cardiomyocytes, and Complex II is exquisitely sensitive to loss of NRF-1 gene expression.

**SDH Subunit Gene Expression**—The effects of NRF-1 silencing on succinate oxidation and the association of Complex II activity with HIF-1α stabilization implicated NRF-1 in the prevention of pseudo-hypoxia through transcriptional regulation SDH, for which all four subunits are nuclear-encoded. On the basis of a bioinformatics promoter analysis, we focused on NRF-1 (and did not investigate NRF-2); moreover, NRF-1 is not up-regulated by mild hypoxia, and the NRF-1 promoter does not contain a canonical HIF-1 consensus motif. Using RT-PCR, we evaluated the extent to which NRF-1 regulates steady-state mRNA levels for SDHα, -b, -c, and -d in H9c2 cardiomyocytes and whether suppressing it deregulates one or more subunits. As a positive control, DCM/CO (50–100 μM) was used to stimulate NRF-1; this increased SDHα mRNA content 3-fold in cardiomyocytes, but SDHb, -c, and -d mRNA less than 2-fold (Fig. 2A). siNRF-1 transfection suppressed mRNA expression for two of four SDH subunits: a and d, for 48 h (Fig. 2B; 18 S rRNA is the densitometry reference for subunit mRNA) but the effect was far greater for SDHα mRNA (~17-fold). This implies a large dynamic range for NRF-1 in regulating the catalytic flavoprotein that converts succinate to fumarate in Complex II.

**NRF-1 Silencing Increases Nuclear HIF-1α Accumulation and Arrests Cardiomyocyte Differentiation**—Studies were done under aerobic conditions to measure the influence of NRF-1 on cell fate determination (Table 1). The use of DCM/CO (50–100 μM) for 24 h to induce NRF-1 (positive control) was sufficient to increase nuclear HIF-1α expression 3-fold in H9c2 cells after DCM treatment for 24 h to induce NRF-1 (positive control). The effects of NRF-1 silencing on nuclear HIF-1α expression were measured with siNRF-1 transfection (Fig. 1). Nuclear HIF-1α was observed in control cells (Fig. 1B, lane 1) and was increased 2-fold by DCM/CO (Fig. 1B, lane 2). Nuclear HIF-1α expression was suppressed by siNRF-1 transfection (Fig. 1B, lane 3). Nuclear HIF-1α expression was suppressed by siNRF-1 transfection (Fig. 1B, lane 3).

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HIF-1 without the O₂ limitation of hypoxia. Aerobic H9c2 cells transfected with siNRF-1 had significant increases in nuclear HIF-1α protein (Fig. 2C). By comparison, DCM/CO, which inhibits cytochrome c oxidase and stimulates mitochondrial ROS in these cells, had the converse effect on nuclear HIF-1α.

After serum removal, embryonic H9c2 rat heart cells proliferate in high serum medium and differentiate over 96 h. Here the use of siNRF-1 inhibited myotubule formation (not shown), thus making it possible that HIF-1α behaves differently in differentiated myocytes. In primary rat cardiomyocytes, nuclear HIF-1α nuclear protein content was stimulated 10-fold by DCM/CO for 24 h or transfected with 80 μM siNRF-1, scRNA, or exposed to DCM/CO (100 μM) for 24 h. Tubulin is a loading control. D, knockdown experiments with siRNA targeting the open-reading frames of NRF-1 and SDHa in rat cardiomyocytes. Western blots for nuclear HIF-1α and NRF-1 protein are compared with tubulin and SDHa with β-actin. Lane 1, protein expression in control cells. Lane 2, protein expression 24 h after DCM/CO used as a positive control to induce NRF-1. Lane 3, SDHa siRNA (80 μM) increases HIF-1α in cardiomyocytes, but has no effect on NRF-1 and decreases SDHa protein expression. Lane 4, NRF-1 siRNA (80 μM) increases HIF-1α and decreases NRF-1 and SDHa protein expression. Lane 5, scrambled siRNA has no effect on HIF-1α, NRF-1, or SDHa protein expression.

Identification of NRF-1 Recognition Sites in Rat SDH Subunit Promoter Regions—To identify NRF-1-binding motifs in the four SDH subunit genes, the rat SDHa, -b, -c, and -d promoters were cloned and sequenced (Fig. 3A). A search of the proximal SDHa promoter for transcription factor-binding sites demonstrated multiple and tandem consensus NRF-1 recognition sites. The rat SDHb promoter revealed two NRF-2 recognition sites and a partial but not a consensus NRF-1 site, and the SDHc promoter revealed no NRF-1, one NRF-2, and multiple tandem Sp1 recognition sites as well as a USF-1 consensus domain (not shown). The proximal SDHd promoter region revealed one NRF-1 and three tandem NRF-2 recognition sites. The NRF-1-binding sites in the SDHa and -d promoters are located within 150 bp of the transcription start site, while the SDHc promoter has three upstream Sp1 sites flanked by NRF-2 sites. A similar configuration of Sp1 and NRF sites has been observed in other genes involved in respiratory protein expression. The five NRF-1 sites are nearly complete matches for canonical NRF-1 binding and contain the invariant nucleotide consensus motif for functional NRF-1 recognition (24).

In Vivo SDHa, -b, -c, and -d Promoter Binding by NRF-1—The ChIP assay was performed to evaluate NRF-1 binding to SDH gene promoters in the H9c2 cardiomyocyte in vivo (Fig. 3B). Fig. 3B demonstrates that NRF-1 binds specifically to the promoter regions of both SDH a and d. When the ChIP is performed after siNRF-1, these promoter fragments are no longer detected by PCR, and no NRF-1-dependent products are obtained with primers for SDHc promoter seg-
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**A**

-180 CAGGCCGGAA CAGGCCGGAA CAGGCCGGAA CAGGCCGGAA CAGGCCGGAA
-121 ACACCCACTG ACGGCAAGCA GGAGGAGGCT GCGGCCAGGC GCGGCCAGGC
-61 ACACCCACTG ACGGCAAGCA GGAGGAGGCT GCGGCCAGGC GCGGCCAGGC

+1 atggccgggct ttcgagcaact ttcgagcaact ttcgagcaact

**B**

| ScRNAs | DCM | SDHa | SDHb | SDHc | IGF | Inputs |
|--------|-----|------|------|------|-----|--------|
|         |     |      |      |      |     |        |
O$_2^-$ production, but this was enhanced by DCM/CO, the positive control (Fig. 7). In contrast, cells treated with either siSDHa or siNRF-1 did not exhibit measurable mitochondrial O$_2^-$ production after 24 h. Thus, silencing functional Complex II in these cardiac cells did not enhance mitochondrial O$_2^-$ release, and mitochondrial ROS production did not initiate HIF-1$\alpha$ nuclear translocation.

**DISCUSSION**

The nuclear transcriptional program for the expression of the proteins of the five respiratory complexes requires NRF-1 (1, 31, 32). NRF-1 is also necessary for mitochondrial biogenesis, has a role in cell growth control (7), and its expression and activity are repressed by cyclin D1 (33). NRF-1 is also necessary for the expression of the mitochondrial transcription factor (Tfam) (2), the TFBI1M and M2 isoforms (34) and RNA-processing proteins required for mtDNA transcription and replication (24). Here we find that NRF-1 silencing functionally blocks succinate oxidation in aerobic heart cells prior to NADH-linked substrate, and that loss of NRF-1 expression increases HIF-1$\alpha$ stability and glucose transporter and glycolytic gene expression characteristic of pseudo-hypoxia.

The regulation of Complex II activity by NRF-1 and nuclear HIF-1$\alpha$ accumulation was a point of focus because of the emerging information on the role of SDH in tumorigenesis. Until now, little was known about NRF-1 regulation of the four nuclear genes of Complex II; SDHa, SDHb, SDHd, and SDHd encode for the hydrophilic flavoprotein (Fp) and iron-sulfur protein, and two hydrophobic membrane anchor subunits, respectively, and SDH activity is integral to both the Krebs cycle and the respiratory chain by coupling matrix succinate to fumarate with the flavin adenine dinucleotide–driven reduction of ubiquinone at the inner membrane (35). The hydrophobic membrane anchor subunits contain heme $b$ and the ubiquinone–binding site, and the centers are arranged to avoid ROS generation by FADH$_2$ in the aerobic state (35, 36).

The evidence that NRF-1 regulates SDHa stems from the multiple NRF-1 but no NRF-2 recognition sites in the proximal nucleotide sequence of the rat gene promoter. NRF-1 silencing

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**FIGURE 4.** Knockdown and transfection experiments in H9c2 cells using siRNA to sequences in the open-reading frame of SDHa mRNA. A, RT-PCR for SDHa normalized to 18S rRNA. Lanes 1 and 2 shows SDHa mRNA expression in control cells. Lanes 3, 4, lanes 5, 6, and lanes 7, 8 show effects of SDHa siRNA (80 $\mu$M) on mRNA expression at 24, 48, and 72 h, respectively. The histogram represents SDHa induction by real time PT-PCR ($n = 3$, *, $p < 0.05$ compared with control). B, Western blotting for mitochondrial succinate-ubiquinol oxidoreductase (SDH) subunit A and B proteins, the two functionally active subunits of Complex II. The blot shows controls (lanes 1 and 2) and cells transfected with siRNA to the SDHa subunit (lanes 3–8). Porin is used as a loading comparison. Lanes 3–6 show SDHa siRNA transiently inhibits SDHa but not SDHb protein expression. C, in-gel quantification of Complex II enzyme activity in control and SDHa-silenced H9c2 cells at two concentrations of SDHa siRNA (40 and 80 $\mu$M). Native gels were incubated in reaction buffer containing 10 mM succinate and developed with nitroblue tetrazolium.

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**D**

NRF-1 and SDHa Regulation

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**C**

SiSDHa

0 40 $\mu$M

80 $\mu$M

SDH Activity

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**B**

SiSDHa

0 24 48 72h

Fig. 6A, and the nuclear accumulation of HIF-1$\alpha$ by Western blot analysis (Fig. 6B). In control studies, $\alpha$-KG did not alter total superoxide dismutase (SOD) activity in SDHa-silenced cells (not shown). Therefore, PHD substrate/product equilibrium is seen as a key regulator of HIF-1$\alpha$ nuclear translocation after SDHa suppression.

**Mitochondrial Superoxide and Nuclear HIF-1$\alpha$ in Aerobic Cells**—HIF-1$\alpha$ nuclear translocation may occur after PHD inhibition by ROS, e.g. superoxide (O$_2^-$) and/or H$_2$O$_2$. Therefore, the mitochondrial ROS leak rate was evaluated in control and SDHa-silenced aerobic H9c2 cells media using fluorogenic mitoSOX dye to track mitochondrial O$_2^-$ generation. Using mitoSOX, control H9c2 cells showed minimal mitochondrial
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abrogates SDHa and SDHd mRNA expression in cardiomyocytes with minimal effect on SDHb or SDHc mRNA levels. ChIP demonstrated strong NRF-1 binding to the SDHa promoter, less binding at SDHd, and no or equivocal binding at SDHc and SDHb. Thus, the proclivity of NRF-1 to regulate HIF-1α stability via SDH promoter binding should be greatest at the flavoprotein, although this does not categorically exclude secondary ETC regulatory sites for the NRF-1 effect.

Although mitochondria are vital for energy provision, they regulate other processes such as apoptosis; moreover, altered mitochondrial function contributes to proliferation in a variety of tumor lines (37, 38) and the loss of SDH activity activates HIF-1 in tumor cells (13). Here in normal heart cells, disruption of a transcriptional pathway in mitochondrial biogenesis causes nuclear HIF-1α accumulation and directly links SDH to HIF-1α stability through PHD inhibition. The aerobic suppression of NRF-1 and loss of SDH activity emulates succinate accumulation in hypoxia, a conserved phylogenetic sensor of O2 deficiency in both invertebrate and vertebrate organisms (39).

A diagram of the pathway for NRF-1 and SDHa regulation of HIF-1 gene expression in cardiac cells is provided in Fig. 8. When NRF-1 is down-regulated, SDHa expression is lost, and succinate oxidation is abrogated, thereby blocking FAD-linked electron entry into the respiratory chain. This decreases PHD activity, HIF-1α escapes degradation, and HIF-1α nucleoprotein increases. SDHa overexpression with siNRF-1 reduces HIF-1α stability and demonstrates independent and sufficient which has implications for NRF-1 as a tumor suppressor, HIF-1 persistence is arguably tumorigenic, e.g. in neuroendocrine tissues.

Because the energy demand of the heart is met principally by aerobic ATP synthesis, it is especially vulnerable to hypoxia, and cardiac cells respond exquisitely to effectors of O2 sensing. Although HIF-1α is a “master” switch in the mammalian hypoxic response, it is also activated by aerobic stressors, and here in aerobic cardiomyocytes, loss of NRF-1 up-regulates HIF-1-responsive genes including selected glucose transporters, hexokinase, and HO-1 (44, 45), congruent with glycolytic protection of rat cardiomyocytes from hypoxic induction of apoptosis (46). Mitochondrial damage also eliminates non-via-ble cells by initiating intrinsic apoptosis (47) and resistance to apoptosis is sensitive to changes in anti-apoptotic protein expression (48, 49). In terms of energy provision, ATP depletion alone does not stabilize HIF-1α and singular Complex II inactivation has more limited consequences than hypoxia or defects in Complex I.

Mitochondrial ROS production was checked as a mechanism by which NRF-1-induced loss of SDH could produce pseudo-hypoxia. In the ETC, ROS generation occurs notably at Complex III, but in theory could arise from defective SDH assembly, or via the SDHa, as in certain bacteria, by conversion to a fumarate reductase (36). That conversion, however, requires FAD binding and inhibition of electron flow from SDHα to iron-sulfur in SDHb and to the inner membrane ubiquinone-binding site. In aerobic cardiac cells, mitochon-

FIGURE 5. SDHa silencing increases HIF-1 target gene expression in H9c2 cells. A, Western blot analysis of glucose transporter proteins Glut1, Glut2, and Glut4 in control lysate (lanes 1 and 2) or lysate from cells transfected with siRNA to SDHa. Lanes 3, 4, lanes 5, 6, and lanes 7, 8 show timed effects of SDHa siRNA on transporter levels. Tubulin is the loading control. The histogram shows Glut 1, 2, and 4 protein levels by Western blot analysis by 24 h. In duplicate experiments, densitometry indicated that both proteins increased after siNRF-1 by an amount comparable to siSDHa (c). B, Western blot analysis for HO-1 in H9c2 cell lysates described in A shows protein induction after SDHa silencing. (Tubulin is for comparison.) The histogram shows relative HO-1 induction by densitometry (n = 3; * indicates p < 0.05 compared with control and Glut1). C, siNRF-1 causes the expected increases in Glut4 and HO-1 protein by Western blot analysis by 24 h. In duplicate experiments, densitometry indicated that both proteins increased after siNRF-1 by an amount comparable to siSDHa (−2-fold and 3-fold respectively; p < 0.05 for both).
Mitochondrial \( O_2^- \) production did not account for HIF-1\(\alpha\) stabilization by either mechanism. Nuclear HIF-1\(\alpha\) is increased by SDHa silencing, but not by an approach that blocks Complex IV and generates mitochondrial superoxide at Complex III (Fig. 7) (25). In fact, as \( P_O2 \) rises in the cell this would actually oppose HIF-1\(\alpha\) stabilization. Nuclear translocation of HIF-1\(\alpha\) in SDHa-silenced cells is also opposed by \( \alpha-KG \) independently of ROS, thereby excluding mitochondrial ROS within the assay limits as a factor in HIF-1\(\alpha\) accumulation after aerobic silencing of SDHa.

That SDHa silencing did not enhance the mitochondrial ROS leak rate in cardiomyocytes concurs with the work of Selak et al. (12, 50) in aerobic tumor cells where \( SDHd \) silencing induces HIF-1 stabilization via succinate accumulation with no detectable increase in ROS. There are other possibilities however; Guzy et al. (53) reported that SDHb silencing increased ROS in aerobic tumor cells and stabilized HIF-1 in a ROS-dependent manner. As in our normal heart cells, SDHa suppression did not increase ROS levels, but in contrast, did not increase HIF-1 stabilization. Guzy et al. (53) postulate that SDHb silencing allowed \( H_2O_2 \) to escape the mitochondria and inhibit PHD. The tumor cell differences reported by Selak and Guzy could be due to methodological or to cell-specific effects, but Guzy et al. (53) did not exclude that HIF-1 accumulation associated with loss of SDHs or \( SDHd \) operates differently than loss of SDHb. Moreover, the importance of succinate in HIF-1\(\alpha\) stabilization is agreed to be sufficient for HIF-1 nuclear translocation in many Complex II activity-deficient tumor cell lines (51).

The product/substrate equilibrium is an important factor in PHD regulation (12, 14) because loss of SDH activity allows succinate accumulation to feedback-inhibit PHD (14), a parsimonious explanation for HIF-1\(\alpha\) nuclear translocation. HIF-1\(\alpha\) PHD1–3 reside in the cytoplasm, but mitochondrial succinate moves via inner membrane dicarboxylate translocators and outer membrane voltage-dependent anion channels to the cytoplasm (52) where it inhibits the forward HIF-1\(\alpha\) hydroxylation reaction because as \( \alpha-KG \) dicarboxylate dioxygenases, the PHDs release succinate upon converting HIF-1\(\alpha\) prolyl to the hydroxyprolyl residues. Thus, equilibrium reversal of nuclear HIF-1\(\alpha\) accumulation by \( \alpha-KG \) connects succinate to activity control of PHD in cardiomyocytes.

In summary, NRF-1 contributes to cytoplasmic HIF-1\(\alpha\) instability in aerobic cardiomyocytes by functional occupancy of two of four subunit gene promoters of cardiac mitochondrial Complex II. Specifically, NRF-1 exquisitely regulates the expression levels of the catalytic SDHa flavoprotein required for
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**FIGURE 8. Diagram of the effects of NRF-1 on the regulation of SDHa and HIF-1α-responsive genes.** The loss of NRF-1 leads to loss of catalytic activity of SDHa and depressed Complex II activity, which causes mitochondria to release succinate. Succinate inhibits the PHD, and HIF-1α escapes the von Hippel-Lindau protein (pVHL) to translocate to the nucleus where it engages with HIF-1α in DNA binding and gene activation. Heme oxygenase-1 (Hmox-1, HO-1) is shown as an example of an HIF-1α-responsive gene.

electron transport into the respiratory chain. NRF-1-dependent loss of SDHa expression allows succinate to accumulate, which promotes nuclear HIF-1α translocation by blocking α-ketoglutarate utilization by the HIF-1 PHD. These findings demonstrate new biological importance for NRF-1 both in the close transcriptional regulation of the SDHa flavoprotein and the avoidance of pseudo-hypoxic gene expression in aerobic cells.

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