Respiratory Uncoupling Induces δ-Aminolevulinate Synthase Expression through a Nuclear Respiratory Factor-1-dependent Mechanism in HeLa Cells*

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Nuclear respiratory factor (NRF)-1 appears to be important for the expression of several respiratory genes, but there is no direct evidence that NRF-1 transduces a physiological signal into the production of an enzyme critical for mitochondrial biogenesis. We generated HeLa cells containing plasmids allowing doxycycline-inducible expression of uncoupling protein (UCP)-1. In the absence of doxycycline, UCP-1 mRNA and protein were undetectable. In the presence of doxycycline, UCP-1 was expressed and oxygen consumption doubled. This rise in oxygen consumption was associated with an increase in NRF-1 mRNA. It was also associated with an increase in NRF-1 protein binding activity as determined by electrophoretic mobility shift assay using a functional NRF-1 binding site from the δ-aminolevulinate (ALA) synthase promoter. Respiratory uncoupling also caused a time-dependent increase in protein levels of ALA synthase, an early marker for mitochondrial biogenesis. ALA synthase induction by respiratory uncoupling was prevented by transfecting cells with an oligonucleotide antisense to the region of the NRF-1 initiation codon; a scrambled oligonucleotide with the same base composition had no effect. Respiratory uncoupling increases oxygen consumption and lowers energy reserves. In HeLa cells, uncoupling also increases ALA synthase, an enzyme critical for mitochondrial respiration, but only if translatable mRNA for NRF-1 is available. These data suggest that the transcription factor NRF-1 plays a key role in cellular adaptation to energy demands by translating physiological signals into an increased capacity for generating energy.

Tissues adapt to an increased need for energy by increasing mitochondria, resulting in an enhanced capacity to generate ATP by oxidative phosphorylation. Examples of this phenomenon include the adaptation of skeletal muscle to exercise (1, 2) and the adaptation of liver to thyrotoxicosis (3, 4). Respiration, the transfer of electrons from fuels to oxygen, provides most of the energy for mammalian cells. This electron transfer is accompanied by respiratory cytochromes at the mitochondrial inner membrane that contain heme (5). δ-Aminolevulinate (ALA)1 synthase is rate-limiting for the synthesis of heme (6). ALA synthase catalyzes the reaction of succinyl-CoA with glycine to form ALA, a precursor for protoporphyrin IX. Protoporphyrin combines with iron to form heme, essential for electron transfer and energy generation. Therefore, the induction of ALA synthase expression is critical to effect an increase in cellular respiratory capacity.

Increasing respiratory capacity requires an increase in mitochondrial size and number. Mitochondria have their own genome, a 16.5-kilobase circular strand of DNA encoding only 13 of the more than 100 proteins necessary for electron transfer to oxygen (5). The remaining proteins necessary for oxidative phosphorylation (including ALA synthase), the enzymes for fatty acid oxidation and the tricarboxylic acid cycle, and the factors regulating mitochondrial DNA transcription and replication are products of nuclear genes. Mitochondrial biogenesis thus depends on the coordination of nuclear and mitochondrial events.

Nuclear respiratory factor (NRF)-1 may be responsible for this coordination. NRF-1, a transcription factor encoded by nuclear DNA (human chromosome 7, Ref. 7), was first identified by Evans and Scarpulla (8) as an activator of cytochrome c gene transcription. Functional binding sites for NRF-1 were subsequently described in several nuclear genes critical for mitochondrial biogenesis, including ALA synthase (9) and human mitochondrial transcription factor A (TFAM) (10). Disruption of the Tfam gene in mice abolishes oxidative phosphorylation and prevents mitochondrial biogenesis (11).

There is no direct evidence that NRF-1 transduces physiological signals to mitochondria. We addressed the question of whether an increase in cellular metabolism prompts an increase in mitochondrial biogenesis through NRF-1. HeLa cells, the source for the initial purification of the NRF-1 protein (12), were engineered to inducibly express uncoupling protein (UCP)-1, and ALA synthase protein was assayed as a marker for stimulation of the assembly of mitochondrial respiratory complexes. The results suggest that NRF-1 is a critical component of the energy-sensing mechanism in mammalian cells.

EXPERIMENTAL PROCEDURES

Cloning of the Mouse UCP-1 cDNA—Mouse UCP-1 was cloned by RT-PCR using brown adipose tissue from cold-induced mice as the source of mRNA. Adult C57BL/6j mice were placed at 4 °C for 6 h (13). Interscapular brown adipose tissue was harvested, total RNA was prepared by equilibrium centrifugation in cesium chloride (14), and poly(A)1 RNA was isolated using reagents in kit form (QIAGEN, Valencia, CA). RT-PCR was performed using avian myeloblastosis virus

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¶ The abbreviations used are: ALA, δ-aminolevulinate; UCP, uncoupling protein; NRF, nuclear respiratory factor; RT-PCR, reverse transcriptase-polymerase chain reaction; Dox, doxycycline; GAPDH, glyceraldehyde-phosphate dehydrogenase; CMV, cytomegalovirus; TRE, tetracycline-responsive element.
reverse transcriptase (Titan™, Roche Molecular Biochemicals). Primers were based on a mouse UCP-1 sequence (nucleotides 184–207 and 1184–1207; GenBank™ accession no. U63419). The upstream primer was 5′-TGA GTC CTT GAA TTC TGG CAC TCA-3′ (with underlined bases indicating substitutions generating an EcoRI site), and the downstream primer was 5′-GTC TCG CAT TGG AGA AGC CCA ATG-3′ (with underlined bases generating an XbaI site). Experiments with mice were approved by the Animal Studies Committee at Washington University.

**Engineering HeLa Cells for Inducible Expression of UCP-1—Regulated expression of UCP-1 was achieved using a doxycycline-inducible gene expression system (Tet-On, CLONTECH, Palo Alto, CA) originally described by Gossen et al. (15). UCP-1 cDNA generated by RT-PCR was directionally cloned into the EcoRI/XbaI sites of the plasmid pTRE and sequenced in entirety. The product, pUCP-1 (Fig. 1), was cotransfected with a plasmid carrying a hygromycin resistance cassette (the ratio of pUCP-1 to the hygromycin resistance plasmid was 20:1) into HeLa cells stably transfected with the pTet-On plasmid (Fig. 1) by calcium phosphate precipitation. Stable transfectants were identified by selection with hygromycin at a concentration of 200 μg/ml. Three independent cell lines stably transfected with mouse UCP-1 (as verified by RT-PCR in the presence of doxycycline) were used for these experiments, and each showed the same results.

**HeLa Cell Culture—** After expansion, aliquots of stably transfected UCP-1 HeLa cells were frozen until needed for specific experiments. Cells were treated with trypsin-EDTA, washed, resuspended in cell freezing medium (10% MeSO, 50% fetal bovine serum, and 40% culture medium (described below)) at 4 °C, kept at −20 °C for 2 h and −80 °C overnight, and then transferred to liquid nitrogen. Aliquots were thawed rapidly at 37 °C and plated in culture medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin). Medium was changed every 3–4 days. To induce UCP-1 expression, cells were fed fresh culture medium, and 24 h later the medium was replaced with culture medium containing either 2 μg/ml doxycycline (+Dox) or an equal volume of carrier (−Dox).

**Oxygen Consumption—** Oxygen consumption of UCP-1 stably transfected HeLa cells treated with doxycycline and mouse brown fat was measured using a model 5300 oxygen monitor (YSI Inc.). In preliminary experiments, oxygen uptake was shown to be linearly dependent on input cellular protein. After 6 h in the presence (+Dox) or absence (−Dox) of 2 μg/ml doxycycline, cells were trypsinized, washed, resuspended in prewarmed culture medium, and placed in magnetically stirred sample chambers containing Clarke-type polarographic oxygen probes. Oxygen consumption was continuously monitored for 15 min for each sample, and data were expressed by normalizing to input cell protein.

For measurement of oxygen consumption in brown fat, three adult C57BL/6J mice were placed at 4 °C for 4.5 h followed by isolation of interscapular brown fat in the following medium: 20 mM potassium phosphate (pH 7.4), 20 mM potassium chloride, 1.6 mM EDTA, 5 mM magnesium chloride, 1 mM sodium malate, 10 mM sodium pyruvate, 123 mM sucrose, and 2 mM Tris (pH 7.4). Brown fat was also isolated from three littermates kept at room temperature. Oxygen consumption was measured as described above.

**Detection of UCP-1, β-Actin, NRF-1, and GAPDH Messages by RT-PCR—** For UCP-1, primers were based on nucleotides 395–970 (GenBank™ accession no. L22445): 5′ CCT CCA TGA AGT TCT ACA 3′. The control “scrambled” phosphorothioate-modified oligonucleotide (with the same base composition as the antisense oligonucleotide) was 5′ CAT GTA CGC AAC TCT ACT 3′.

HeLa cells containing pUCP-1 were transfected with antisense or scrambled oligonucleotides using cationic liposomes (LipofectAMINE, Life Technologies, Inc.) by a modification of the protocol described by Quaggin et al. (17). 30 μg of liposomes were complexed with antisense or scrambled oligonucleotide and then added to 5 ml of prewarmed culture medium containing 2 μg/ml doxycycline to yield a final oligonucleotide concentration of 1 μg/ml.

For antisense experiments, cells were fed culture medium containing 2 μg/ml doxycycline to initiate transcription of the UCP-1 gene. After 20 min, the medium was replaced with medium containing 5 μg/ml doxycycline and either NRF-1 antisense or scrambled oligonucleotide complexed with cationic liposomes. 16 h later, cells were harvested, extracts were prepared, and ALA synthase protein was assayed by Western blotting.
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RESULTS

HeLa cells were isolated that contained both pTet-On (Fig. 1A), directing constitutive expression of a transcriptional activator that binds to a tetracycline-responsive element (TRE) in the presence of doxycycline, and pUCP-1 (Fig. 1B), which contains a TRE. In the absence of doxycycline, control human ß-actin message was detected in pTet-On/pUCP-1 HeLa cells, but there was no expression of UCP-1 mRNA in multiplex RT-PCR assays of total RNA (Fig. 2A, −Dox). After treatment with 2 μg/ml doxycycline for 6 h, the same cells expressed UCP-1 mRNA (Fig. 2A, +Dox). Protein levels mirrored mRNA expression. The 32-kDa UCP-1 protein was undetectable in extracts from cells cultured in the absence of doxycycline but present when parallel cultures of the same cells were treated with 2 μg/ml doxycycline for 6 h (Fig. 2B). Induction of UCP-1 expression by doxycycline increased oxygen consumption (Fig. 3A). The maximum effect was observed at 6 h. At this time point, HeLa cells carrying pUCP-1 utilized twice as much oxygen as untreated cells (8.37 ± 1.19 versus 4.37 ± 0.36 nmol/min/mg protein, mean ± S.E., p = 0.0007, 4 experiments). Doxycycline treatment of HeLa cells carrying only pTet-On (Fig. 3A, first two bars from the left) had no effect on oxygen consumption.

To compare the physiological properties of HeLa cells to brown fat, mice were exposed to cold (4 °C for 4.5 h) followed by isolation of brown fat and determination of its oxygen consumption and UCP-1 protein content. Cold exposure increased oxygen consumption by 52% and UCP-1 expression by 62% in brown fat (Fig. 3B). Oxygen consumption in doxycycline-treated HeLa cells was similar to that of brown fat (54% or 8.37 pmol/min/mg protein, mean ± S.E.) whereas UCP-1 expression was proportionally lower in HeLa cells. In a typical experiment using equal amounts of protein, UCP-1 protein was 10.7 densitometry units in HeLa +Dox compared with 49.6 units in 24 °C brown fat. However, UCP-1 expression was proportionally lower in HeLa cells. In a typical experiment using equal amounts of protein, UCP-1 protein was 10.7 densitometry units in HeLa +Dox compared with 49.6 units in 24 °C brown fat. Like messages for many transcription factors, NRF-1 mRNA was not abundant. NRF-1 mRNA was assayed semiquantitatively as described under “Experimental Procedures.” cDNA synthesized from HeLa RNA was serially diluted and then subjected to PCR under conditions within the linear response range of the assay. As shown in Fig. 4, the NRF-1 mRNA was higher in the setting of UCP-1 induction for 16 h (+Dox), as compared with cells not expressing UCP-1 (−Dox), whereas there was no effect of uncoupling on GAPDH mRNA levels. This is best seen by comparing lanes 4 and 9, which contain products from template that was diluted 16-fold. The RT-PCR signals from Fig. 4A are graphically displayed in Fig. 4B. For NRF-1, there is a 2.4-fold difference (p = 0.0046) between the slopes of the +Dox line (r = −0.9857) and −Dox line (r = −0.9843). The same results were seen in three experiments.

NRF-1 protein was undetectable by Western blotting. However, NRF-1 protein binding (Fig. 5) was detected by electrophoretic mobility shift assay using an oligonucleotide containing a functional NRF-1 binding site from the ALA synthase promoter and HeLa nuclear extracts. The NRF-1-specific band is indicated by the asterisk in Fig. 5A. This band was absent from lanes containing probe but no extract (lane 1). It also did not appear in gel shift assays performed using negative control probes.
oligonucleotides containing a mutated NRF-1 binding site (data not shown, see “Experimental Procedures” for the sequence of the negative control oligonucleotides). The band was supershifted to the level of the arrow in panel A when reactions included an NRF-1 antibody (lane 4) but not when they were performed in the presence of an antibody to ALA synthase (lane 3).

NRF-1 gel shift activity was greater when respiration was uncoupled by doxycycline (Fig. 5B). The asterisk in Fig. 5B indicates the position of the same band identified by the asterisk in Fig. 5A. The NRF-1 gel shift was more intense in the presence of doxycycline (Fig. 5B, lane 2) as compared with -Dox cells (lane 1), and the signal was essentially abolished by including a 10-fold molar excess of unlabeled probe (lane 3). Radiographic density comparisons were made using data from experiments conducted under conditions of probe excess and

FIG. 3. Induction of UCP-1 expression increases oxygen consumption. A, after 6 h in the presence (+Dox) or absence (-Dox) of 2 μg/ml doxycycline, HeLa cells containing both pTet-On and pUCP-1 (HeLa-UCP-1) or only pTet-On (HeLa) were placed in magnetically stirred sample chambers equipped with Clarke-type oxygen probes. Oxygen consumption was linearly dependent on input cell protein. **, p = 0.0007 versus -Dox, 4 experiments. B, mice were kept at room temperature (24 °C) or placed at 4 °C for 4.5 h followed by isolation of brown fat tissue from the interscapular area. Tissue was assayed for oxygen consumption (left side of panel) using a Clarke-type probe and for UCP-1 protein content (right side of panel) by Western blotting. For oxygen consumption in B, **, p = 0.0320 versus 24 °C. For UCP-1 protein in B, **, p = 0.0006 versus 24 °C.
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Fig. 5. Respiratory uncoupling increases NRF-1 binding activity in HeLa cells. HeLa cells were cultured in the presence (+Dox) or absence (−Dox) of doxycycline for 16 h followed by the preparation of nuclear extracts. Electrophoretic mobility shift assays were performed using an oligonucleotide containing a functional NRF-1 binding site from the ALA synthase (ALAS) promoter. The position of the specific gel shift is indicated by the asterisk in A. No binding was seen at this position using control oligonucleotides (not shown). The lower radioactive bands represent nonspecific binding. Free probe was run off the bottom of this gel. The position of the supershift in the presence of an NRF-1 antibody is indicated by the arrow. The intensity of the NRF-1 gel shift (indicated by the asterisk) is greater in the presence (lane 5), as compared with the absence (lane 2), of doxycycline in this overexposed gel. B shows a shorter exposure of a different experiment comparing the intensity of the NRF-1 gel shift in the presence (lane 2) and absence (lane 1) of doxycycline. The asterisk indicates the same gel position identified by the asterisk in A. Oligo refers to the absence (lanes 1 and 2) or presence (lane 3) of a 10-fold molar excess of unlabeled oligonucleotide probe. C shows densitometric quantitation of NRF-1 gel shifts from several experiments in the absence (−Dox) and presence (+Dox) of doxycycline. *, p = 0.0047, 4 experiments. All binding studies were done in the presence of probe excess and within the linear response range of the gel shift assay. EMSA, electrophoretic mobility shift assay.

Fig. 6. Respiratory uncoupling increases ALA synthase expression but not in the presence of an NRF-1 antisense oligonucleotide. HeLa cells were cultured in the presence (+Dox) or absence (−Dox) of doxycycline for 0–16 h followed by preparation of cellular extracts. Extracts were subjected to Western blotting using rabbit anti-ALA synthase antisera. A shows a representative experiment after 6 h of antibiotic exposure. B shows the densitometric quantitation of ALA synthase protein in the presence and absence of doxycycline. The first two bars from the right represent cells transfected with an NRF-1 antisense oligonucleotide or a scrambled oligonucleotide (same base composition with a different sequence). ***, p = 0.0005 versus −Dox by two-tail t test, p < 0.001 by Tukey-Kramer test. *, p = 0.0176 versus NRF-1 antisense by two-tail t test, p < 0.05 by Tukey-Kramer test.

Respiratory uncoupling also induced ALA synthase expression (Fig. 6). Treatment with doxycycline for 16 h increased the mass of the 70-kDa ALA synthase protein as assayed by Western blotting (Fig. 6A). The induction of ALA synthase was time-dependent; an intermediate signal was detected after 6 h of doxycycline treatment (Fig. 6B). At 16 h of doxycycline treatment (Fig. 6B, double asterisk), ALA synthase protein was increased 2.2-fold (p = 0.0005 by two-tail t test).

The increase in ALA synthase associated with uncoupling was prevented by transfecting cells with an oligonucleotide antisense to the NRF-1 message. After 16 h of doxycycline treatment, NRF-1 antisense-treated cells did not show an increase in ALA synthase protein (Fig. 6B, second bar from the right), but an induction did occur in cells transfected with a scrambled (same base composition as the NRF-1 antisense oligonucleotide in a different sequence) oligonucleotide (Fig. 6B, asterisk, p = 0.0176 for antisense versus scrambled by two-tail t test).

The relevant comparisons of Fig. 6B remained significant when analyzed by analysis of variance and the Tukey-Kramer multiple comparisons test. In cells treated with doxycycline for 16 h (double asterisk), ALA synthase expression was greater than in −Dox cells at 16 h (p < 0.001) and 0 h (p < 0.001). In cells treated with doxycycline and the scrambled oligonucleotide (asterisk), ALA synthase expression was greater than in cells treated with the NRF-1 antisense oligonucleotide (p < 0.05), −Dox cells at 16 h (p < 0.01), and −Dox cells at 0 h (p < 0.01).

To address the issue of whether the observed increase in ALA synthase is due entirely to an increase in NRF-1, HeLa
cells overexpressing NRF-1 were generated (Fig. 7). Cells were stably transfected with pNRF-1 (containing human NRF-1 cDNA driven by the CMV promoter) and compared with mock-transfected cells. The 68-kDa NRF-1 protein was not detected in three independent mock-transfected HeLa cell isolates but was easily detected in three independent cell lines stably transfected with pNRF-1 (Fig. 7, arrows in inset). ALA synthase protein levels were higher in the cells overexpressing NRF-1 (Fig. 7, p = 0.028). However, the magnitude of the ALA synthase increase (43%) in the setting of high level NRF-1 expression was less than that observed after UCP-1 expression (see Fig. 6), which was associated with low level induction of NRF-1 expression.

**DISCUSSION**

Endurance exercise elevates skeletal muscle respiratory capacity by increasing mitochondrial size and number (1), but the mechanisms that translate an exercise-derived physiological signal into mitochondrial biogenesis are unknown. The current work establishes a system suitable for dissecting the link between energy demands and mitochondrial adaptations to those demands.

Expression of UCP-1 is a suitable mimic of exercise and the stimulation of mitochondrial proliferation. By moving protons from outside to inside the inner mitochondrial membrane without ATP synthesis (21), uncoupling proteins generate heat, decrease energy stores, and increase oxygen consumption, which are classic exercise responses. Exercise causes mitochondrial proliferation in muscle. An unexplained human syndrome of acquired respiratory uncoupling is associated with mitochondrial proliferation in muscle (22). The mitochondrial proliferation that occurs in brown adipose tissue after cold exposure is associated with an increase in UCP-1 and mitochondrial uncoupling (13).

Our results show that inducible expression of UCP-1 results in a 2-fold increase in oxygen consumption (Fig. 3), NRF-1 message (Fig. 4), NRF-1 binding activity (Fig. 5), and ALA synthase protein (Fig. 6A). The induction of ALA synthase protein is prevented by an oligonucleotide antisense to the NRF-1 message (Fig. 6B). These data suggest that increased energy consumption increases expression of the NRF-1 gene, which increases ALA synthase expression by interacting with the NRF-1 binding site in the ALA synthase promoter. Functional binding sites for NRF-1 are also found in the promoters for other genes critical for mitochondrial biogenesis, including human cytochrome c (23), ATP synthase (24), cytochrome oxidase (12), and mitochondrial transcription factor A (10). This observation is consistent with a role for NRF-1 in coordinating energy-related increases in mitochondrial biogenesis.

NRF-1 is not exclusively responsible for the changes in ALA synthase protein observed after UCP-1 expression. High level overexpression of NRF-1 alone increased ALA synthase protein to a lesser degree (Fig. 7) than expression of UCP-1 (Fig. 6). These results suggest that additional transcription factors are necessary to achieve maximal induction of ALA synthase expression or that UCP-1 expression is associated with posttranscriptional mechanisms promoting the accumulation of ALA synthase mass. However, there was no UCP-1-mediated increase in ALA synthase when NRF-1 translation was inhibited using an antisense oligonucleotide. Taken together, these data suggest that NRF-1 is necessary for the induction of ALA synthase expression but not sufficient for maximal expression.

We specifically chose ALA synthase as a marker for activation of mitochondrial function for three reasons. First, exercise is known to increase ALA synthase expression in skeletal muscle (25, 26). Second, ALA synthase, with a half-life estimated at 0.5–2 h (27, 28), has a more rapid turnover rate than other major proteins important for respiration. Even if one assumes a protein half-life of 2 h, 16 h of UCP-1 induction represents 8 half-lives, more than sufficient time for ALA synthase protein mass to reflect a physiological signal. Third, the ALA synthase promoter contains a functional NRF-1 binding site (9).

NRF-1 is a transcription factor belonging to a small family of regulatory proteins important for neuromuscular development in *Drosophila* and sea urchins (29, 30). These proteins share a preserved N-terminal DNA binding domain; phosphorylation of this domain in NRF-1 promotes DNA binding (31). Because exercise stimulates the mitogen-activated protein kinase pathway in skeletal muscle (32) and functional NRF-1 binding sites are found in the promoters of respiratory genes, it is reasonable to implicate NRF-1 in cellular adaptation to energy demands. Electrical stimulation of neonatal cardiac myocytes increases NRF-1 mRNA and increases NRF-1 binding to the cytochrome c promoter (33). An acute bout of exercise transiently increases NRF-1 expression in rat muscle (34). However, no direct data link a physiological signal and induction of a mitochondrial protein through NRF-1. Our work provides that link. Uncoupling, an exercise mimic, induces NRF-1 expression and ALA synthase protein but only when translatable NRF-1 mRNA is available.

The specific signal upstream of NRF-1 that is triggered by UCP-1 expression is unknown. Calcium is a prime candidate. In other cell systems, mitochondrial uncoupling is known to
increase intracellular calcium (35). Calcium-regulated phosphorylation pathways have recently been shown to affect fiber-type specific gene expression, in part through the MEF2 family of transcription factors (36). MEF2C (37) and NRF-1 (31) phosphorylation are strikingly similar, raising the possibility that exercise-induced calcium fluxes alter the activities of these transcription factors through a common mediator.

Our data provide initial evidence that NRF-1 transduces energy-related signals to mitochondria. Future studies will address how NRF-1 senses the energy state of the cell, whether muscle cells respond similarly to uncoupling, and whether NRF-1 alone is sufficient to promote mitochondrial proliferation in the skeletal muscle of animals.

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