Negative feedback maintenance of heme homeostasis by its receptor, Rev-erb α

Nan Wu, Lei Yin, Elysha A. Hanniman, Shree Joshi, and Mitchell A. Lazar¹

Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, Department of Genetics, and The Institute for Diabetes, Obesity, and Metabolism, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, USA

Intracellular heme levels must be tightly regulated to maintain proper mitochondrial respiration while minimizing toxicity, but the homeostatic mechanisms are not well understood. Here we report a novel negative feedback mechanism whereby the nuclear heme receptor Rev-erb α tightly controls the level of its own ligand. Heme binding to Rev-erb α recruits the NCoR/histone deacetylase 3 (HDAC3) corepressor complex to repress the transcription of the coactivator PGC-1 α , a potent inducer of heme synthesis. Depletion of Rev-erb α derepresses PGC-1 α , resulting in increased heme levels. Conversely, increased Rev-erb α reduces intracellular heme, and impairs mitochondrial respiration in a heme-dependent manner. Consistent with this bioenergetic impairment, overexpression of Rev-erb α dramatically inhibits cell growth due to a cell cycle arrest. Thus, Rev-erb α modulates the synthesis of its own ligand in a negative feedback pathway that maintains heme levels and regulates cellular energy metabolism.

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Heme is an essential molecule for many biological functions (Furuyama et al. 2007). In hemoproteins, such as hemoglobin and cytochromes, heme functions as a prosthetic group serving physiological functions as a transporter for oxygen and electrons (Terwilliger 1998). Ironically, heme can cause oxidative stress when it reacts with molecular oxygen, which in turn causes DNA damage, lipid peroxidation, and protein denaturation (Levin et al. 1973; Chiu and Lubin 1989; Vile et al. 1994; Rahman et al. 1997). Consequently, cellular heme homeostasis is tightly controlled at the level of its biosynthesis, which is rate-limited by ALAS1 (δ -aminolevulinic acid synthase 1), as well as its degradation by HMOX1 (Heme oxygenase 1) (Maines 1988; Furuyama et al. 2007). Heme-induced expression of Hmox1 is an acute and robust response, mainly via the function of transcriptional repressor Bach1 (Sun et al. 2002). However, little is known about how physiological concentrations of heme feedback to maintain heme homeostasis.

Circadian rhythm plays an important role for many metabolic processes, such as sleep–wake cycle, blood pressure, and glucose metabolism (Lowrey and Takahashi 2004; Stratmann and Schibler 2006; Kohsaka and Bass 2007). The molecular clock is driven by an interlocked transcriptional and translational feedback loop. BMAL1

and CLOCK, two positive regulators, activate the expression of negative regulators including Cry, Per, and Rev-erb α (Thresher et al. 1998; Lucas and Foster 1999; Preitner et al. 2002; Yin and Lazar 2005). As CRY and PER reach critical levels, they then bind directly to BMAL1/CLOCK and repress their own transcription (Darlington et al. 1998; Shearman et al. 2000; Reppert and Weaver 2001). Rev-erb α directly represses transcription of Bmal1 and therefore serves as an additional feedback loop of circadian circuitry (Preitner et al. 2002; Ueda et al. 2002; Yin and Lazar 2005; Yang et al. 2007). In addition to Bmal1, Rev-erb α regulates metabolic genes, including glucose 6-phosphatase (Yin et al. 2007), ApoCIII (Coste and Rodriguez 2002), and Elov13 (Downes et al. 1995), suggesting that this nuclear receptor (NR) links circadian rhythm to metabolism (Duez and Staels 2008).

Rev-erb α , also known as NR1D1, is a member of the NR superfamily of ligand-regulated transcription factors (Yang et al. 2007; McKenna et al. 2009). Unlike most other NRs, Rev-erb α does not recruit coactivators and functions primarily as a repressor by interacting with the NR corepressor NCoR (Harding and Lazar 1995; Zamir et al. 1996). Recently, we and others identified heme as a physiological ligand for Rev-erb α (Raghuram et al. 2007; Yin et al. 2007; O'Malley 2008). Heme binds reversibly to Rev-erb α and stimulates its interaction with NCoR, enhancing repression of its gene targets (Raghuram et al. 2007; Yin et al. 2007; O'Malley 2008; Rogers et al. 2008).

¹Corresponding author.

E-MAIL lazar@mail.med.upenn.edu; FAX (215) 898-5408.

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Heme biosynthesis is also subjected to circadian control (Zheng et al. 2001; Kaasik and Lee 2004; Burris 2008; Rogers et al. 2008). The mRNA of *ALAS1* shows a robust circadian oscillation (Panda et al. 2002). NPAS2, a homolog of Clock (Dudley et al. 2003), binds to the promoter of *Alas1* and may contribute to the circadian expression of *Alas1* (Kaasik and Lee 2004). PGC-1 α , a key metabolic transcriptional regulator, can induce the expression of *Alas1* in a NRF1- and FOXO1-dependent manner (Handschin et al. 2005). PGC-1 α itself is also subject to circadian regulation (Liu et al. 2007), suggesting it may also be important for driving oscillatory expression of *Alas1* and heme biosynthesis. However, the circadian machinery for PGC-1 α oscillation is unknown.

Here we show that Rev-erb α regulates intracellular heme levels by directly repressing the expression of PGC-1 α via recruitment of NCoR in a heme-responsive manner. Manipulation of heme levels thus regulates PGC-1 α in the direction opposite to that of the change in heme levels in a classic feedback loop. Heme deficiency induced by overexpression of Rev-erb α also restricts mitochondrial respiration-driven oxygen consumption, and therefore causes cell cycle arrest. Together, these data demonstrate the existence of a negative feedback loop that regulates heme levels and represents a novel link between the circadian clock and energy homeostasis.

Results

Manipulation of Rev-erb α changes intracellular heme levels

Feedback regulation by cognate ligands has been shown to be an important mechanism for modulating NR activity (Chin and Gharib 1986; Therrien and Drouin 1993; Chiamolera and Wondisford 2009). Since heme is the physiological ligand for Rev-erb α and its biosynthesis is subjected to circadian control, we postulated that Rev-erb α might directly regulate heme level via a negative feedback loop. We first validated our heme measurement system by examining heme levels in HepG2 human liver cells treated with succinylacetone, a potent inhibitor of heme synthesis (Ebert et al. 1979). Indeed, we observed a dose-dependent drop in the total heme level following succinylacetone treatment (Supplemental Fig. S1). To study the effect of Rev-erb α on heme biosynthesis, we generated cell lines expressing full-length human Rev-erb α under the control of a doxycycline-repressible promoter in NIH3T3 fibroblasts ("REV-3T3 cells"). Since Rev-erb α protein is unstable in many cellular environments, we used a stabilized form (S55D/S59D) to avoid this confounding factor (Yin et al. 2006). The increased expression of total Rev-erb α protein was confirmed by Western blot (Fig. 1A). Remarkably, heme levels were markedly decreased in the REV-3T3 cells (Fig. 1B). In contrast, depletion of Rev-erb α by siRNA led to an increased heme level (Fig. 1C). Efficiency of Rev-erb α knockdown was confirmed by the down-regulation of Rev-erb α mRNA (Fig. 1D). Taken together, these results suggest that Rev-erb α negatively regulates the level of its

Figure 1. Rev-erb α regulates intracellular heme levels. (A) Overexpression of Rev-erb α . Expression level of total Rev-erb α protein in NIH3T3 cells stably transfected control vector (Control-3T3) or vector expressing Rev-erb α SD55/59 (REV-3T3). (B) Rev-erb α reduces heme levels. Intracellular heme levels were measured in NIH3T3 cells stably expressing either control (Control-3T3) or Rev-erb α SD55/59, a stable form of Rev-erb α (REV-3T3). Mean $\pm$ SEM ($n = 3$). (*) $P < 0.05$ versus control cells expressing empty vector. (C) Depletion of Rev-erb α increases heme levels. Intracellular heme levels were measured in either control or Rev-erb α -deficient HepG2 cells. Mean $\pm$ SEM ($n = 3$). (*) $P < 0.05$ versus control shRNA β -galactosidase. (D) Depletion of Rev-erb α . shRNA knockdown of either β -galactosidase (control) or human Rev-erb α in HepG2 liver cells. Rev-erb α mRNA was quantitated by QPCR and normalized to those of GAPDH level. Mean $\pm$ SD ($n = 3$). (*) $P < 0.05$ compared with control by Student's t -test.

own ligand and thus engages in a feedback loop regulating its own activity.

Rev-erb α reduces heme levels by repressing PGC-1 α

We next searched for the molecular target of Rev-erb α regulation of heme metabolism, turning our attention to PGC-1 α , a circadian gene that positively regulates *Alas1* and heme synthesis (Zheng et al. 2001; Panda et al. 2002; Handschin et al. 2005). As expected, heme levels were reduced by depletion of PGC-1 α from HepG2 cells (Fig. 2A). Importantly, under these conditions, depletion of Rev-erb α did not significantly increase heme levels, while the knockdown of Rev-erb α alone increased the heme concentration (Fig. 2A). These data show that elevation of intracellular heme levels by Rev-erb α requires PGC-1 α .

To determine if Rev-erb α directly affects PGC-1 α gene expression, we depleted Rev-erb α in HepG2 human hepatoma cells (Supplemental Fig. S2) and observed a significant increase in the total level of PGC-1 α (Fig. 2B). In addition, using adeno-associated virus (AAV)-mediated transduction, we overexpressed the stabilized form of Rev-erb α in mouse liver (Fig. 2C). PGC-1 α expression was markedly diminished in livers of mice expressing ectopic Rev-erb α (Fig. 2D). Consistent with the role of PGC-1 α in regulating the rate-limiting enzyme

Figure 2. Rev-erb α represses PGC-1 α gene expression. (A) PGC-1 α is required for Rev-erb α regulation of heme levels. HepG2 cells were infected with adenovirus expressing Rev-erb α shRNA and/or PGC-1 α shRNA as indicated. Mean $\pm$ SEM is shown. (*) $P < 0.05$ versus control adenovirus. (B) Rev-erb α depletion induces PGC-1 α . PGC-1 α gene expression was measured after siRNA knockdown of either scrambled sequence (control) or human Rev-erb α in HepG2 liver cells. (C) Overexpression of Rev-erb α . Rev-erb α was determined by QPCR in mouse livers with tail-injected AAV-GFP (control) and AAV-Rev-erb α 55/59SD. (*) $P < 0.05$ versus control. (D) Rev-erb α represses PGC-1 α expression. PGC-1 α and Alas1 were measured in livers from mice transduced by tail injection of AAV-GFP (control) and AAV-Rev-erb α 55/59SD.

in heme biosynthesis, Alas1 mRNA was also down-regulated in the livers with ectopic expression of Rev-erb α .

Rev-erb α directly represses PGC-1 α transcription via functional retinoic acid receptor-related orphan receptor response elements (ROREs)

Analysis of the PGC-1 α gene promoter region up to -3.1 kb from the transcriptional start site failed to identify functional Rev-erb α response elements (Czubryt et al. 2003; data not shown). However, the importance of intronic NR response elements has been increasingly recognized (Carroll and Brown 2006; Carroll et al. 2006; Lefterova et al. 2008; Schupp et al. 2009). Indeed, we identified two putative Rev-erb α monomer-binding sites (ROREs) spaced by 6 base pairs (bp) within the first intron region of the PGC-1 α gene and conserved in human and mouse (Fig. 3A). Rev-erb α overexpression repressed the luciferase activity of a reporter driven by the PGC-1 α intron sequence (Fig. 3B). This repression was dose-dependent, as the increasing amounts of the cotransfected Rev-erb α expression plasmid further reduced the luciferase activity (Supplemental Fig. S3A). Moreover, consistent with the requirement for two Rev-erb α monomers to recruit corepressor and inhibit transcription (Zamir et al. 1997; Yin and Lazar 2005; Wang et al. 2006), mutation of either RORE led to marked reductions in the ability of Rev-erb α to repress the luciferase, and mutation of both sites abolished repression (Supplemental Fig. S3B).

Promoter analysis by chromatin immunoprecipitation (ChIP) in 293T cells revealed the enrichment of Rev-erb α at the implicated region within intron 1 of the endogenous PGC-1 α gene, but not at the negative control (Fig. 3C). Histone deacetylase 3 (HDAC3), which is stoichiometrically associated with NCoR (Guenther et al. 2000; Yoon et al. 2003), was also enriched at the Rev-erb α -responsive region of the PGC-1 α gene (Fig. 3C), and depletion of Rev-erb α by siRNA in 293T cells led to a loss of recruitment of HDAC3 at the intronic Rev-erb α -binding sites of the PGC1 α gene (Supplemental Fig. S4). Furthermore, siRNA depletion of either NCoR or HDAC3 (Supplemental Fig. S5A,B) led to a significant increase in

Figure 3. Rev-erb α recruits the HDAC3/NCoR corepressor complex to repress the PGC-1 α gene through an intronic Rev-erb regulatory element. (A) Schematic presentation of the PGC-1 α Intron1 sequence in which two conserved Rev-erb α -binding monomeric sites are closely located. (ROREd) distal RORE; (ROREp) proximal RORE. (B) Rev-erb α regulation of PGC-1 α intron luciferase reporter transfected in HEK 293T cells. The control is pGL-3 promoter vector. PGC-1 α luciferase reporter plasmid (0.1 μ g) was used in transfection mixture along with 2 μ g of pCDNA-Flag-Rev-erb α expression vector. The luciferase activities of all experiments are expressed as the mean $\pm$ SD ($n = 3$). (C) ChIP assay for recruitment of Rev-erb α and HDAC3 in 293T cells. (D,E) HDAC3 knockdown (D) or NCoR knockdown (E) induces endogenous PGC-1 α gene expression. After shRNA or siRNA transfection, total RNA was prepared and PGC-1 α gene expression was analyzed relative to GAPDH control by quantitative real-time PCR. The fold change was calculated as the relative abundance of PGC-1 α mRNA in the cells receiving HDAC3 shRNA or NCoR siRNA divided by the relative abundance of PGC-1 α mRNA in the cells receiving control shRNA or siRNA, which were set to 1. Results are expressed as mean $\pm$ SD. (*) $P < 0.05$ by paired Student's t -test.

PGC-1 α mRNA (Fig. 3C,D). These data indicate that the basal repression of PGC-1 α by Rev-erb α requires the NCoR/HDAC3 corepressor complex.

Heme enhances Rev-erb α repression of PGC-1 α

Heme binding stimulates NCoR/HDAC3 recruitment and repression by Rev-erb α (Raghuram et al. 2007; Yin et al. 2007). Having shown that Rev-erb α directly represses PGC-1 α , we hypothesized that manipulation of heme levels would regulate PGC-1 α expression. Indeed, depletion of heme by succinylacetone treatment led to a significant induction of PGC-1 α as well as its target, Alas1 (Fig. 4A). In contrast, the addition of heme suppressed PGC-1 α and Alas1 gene expression in human HepG2 cells (Fig. 4B) and mouse primary hepatocytes (Fig. 4C). Additionally, the heme treatment rescued the succinylacetone effect on PGC-1 α (Supplemental Fig. S6B). The repressive effect of heme was abrogated when

Rev-erb α was depleted (Fig. 4D), demonstrating that heme regulation of PGC-1 α is mediated by Rev-erb α . Further supporting this hypothesis, we observed heme-dependent recruitment of NCoR and HDAC3 at the Rev-erb α -responsive region of the endogenous PGC-1 α gene, to which Rev-erb α is constitutively bound (Fig. 4E). Taken together, our results support the hypothesis that heme regulates its own synthesis by stimulating Rev-erb α -mediated repression of the PGC-1 α gene.

Rev-erb α inhibits respiration-driven oxygen consumption rate and mitochondrial gene expression

Since stable expression of Rev-erb α lowers intracellular heme levels, and heme deficiency has been shown to adversely affect mitochondrial complex IV activity (Atamna 2004), we suspected that Rev-erb α might affect mitochondrial function. Indeed, cells expressing Rev-erb α exhibited a lower basal respiration-driven oxygen consumption rate, reflecting a lower basal ATP synthesis rate (Fig. 5A). The negative effect of Rev-erb α on respiration was partially reversed by the addition of heme (Fig. 5B). The same concentration of heme had little effect on cells that did not express ectopic Rev-erb α (Supplemental Fig. S7). The incomplete rescue by heme is likely related to other, direct effects on nuclear genes involved in mitochondrial function that are known to be regulated by PGC-1 α (Wu et al. 1999; Glass 2006). Indeed, the mRNA level of COX5A, IDH3, and MCAD are decreased in REV-3T3 cells, which could partially contribute to the mitochondrial defect in those cells (Fig. 5C).

Overexpression of Rev-erb α causes cell growth arrest

As chronic overexpression of Rev-erb α dramatically reduces respiration-driven oxygen consumption, we next determined the effects of this metabolic change on cell growth. We were particularly interested in this issue because we have been unable to generate and passage cell lines that constitutively express Rev-erb α (data not shown). Indeed, induction of Rev-erb α in the REV-3T3 cells markedly slowed cell growth (Fig. 6A). This reduced rate of growth was reversed when doxycycline was added to stop Rev-erb α expression (data not shown), suggesting that Rev-erb α induced a cell cycle block rather than cell death. Conversely, the depletion of Rev-erb α in NIH3T3 led to increased cell growth (Fig. 6B). Flow cytometry performed on two different subclones of the REV-3T3 cells demonstrated that REV-3T3 cells were arrested in G2/M phase (Fig. 6C; Supplemental Fig. S9A,B).

Discussion

Here we showed that, in addition to regulating circadian rhythms and intermediary metabolism (Preitner et al. 2002; Ueda et al. 2002; Yin and Lazar 2005; Yin et al. 2007; Duez et al. 2008), Rev-erb α reduces the level of its own ligand, heme, in a pathway with the features of a classical negative feedback loop (Fig. 7). This feedback is likely to dampen the magnitude and duration of the Rev-erb α target gene repression. Indeed, we show here that one of

Figure 4. Intracellular heme concentration modulates PGC-1 α gene expression. (A) Succinylacetone induces the expression of PGC-1 α and ALAS1. HepG2 cells were treated with succinylacetone (5 mM) for 16 h. mRNA were quantitated by RT-PCR and normalized to GAPDH. (B) Heme represses Alas1 and PGC-1 α expression in HepG2 cells. Heme treatment was 10 μ M for 16 h. (C) Heme represses Alas1 and PGC-1 α in primary mouse hepatocytes. Heme treatment was 6 μ M for 16 h. (D) Effect of heme on the expression of PGC-1 α gene in cells depleted of Rev-erb α in HepG2 cells. Mean $\pm$ SD ($n = 3$); (*) $P < 0.05$ compared with DMSO-treated cells transfected with control shRNA. (E) Effect of heme on the occupancy of Rev-erb α , HDAC3, and NCoR at the PGC-1 α intronic sequence in HepG2 cells. Results of heme treatment are normalized to DMSO results.

Figure 5. Rev-erb α inhibits respiration-driven oxygen consumption rate and mitochondrial gene expression. (A) Ectopic expression of Rev-erb α inhibits respiration-driven oxygen consumption rate. Oxygen consumption rates and calculations were performed as in the Materials and Methods. Mean $\pm$ SEM ($n = 15$); (*) $P < 0.05$. (B) Addition of heme ($2.5 \mu\text{M}$ for 6 h) partially rescues the inhibition of oxygen consumption by Rev-erb α . Mean $\pm$ SEM ($n = 3$); (*) $P < 0.05$. (C) Mitochondrial gene expression in REV-3T3 cells.

these targets is PGC-1 α , itself a circadian gene that regulates metabolism (Fig. 7). It is the link between Rev-erb α and PGC-1 α that forms the basis of the feedback loop, with heme activating Rev-erb α repression of PGC-1 α , thereby reducing heme synthesis and dampening the extent of Rev-erb α repression. Conversely, when heme is reduced, reduced Rev-erb α repression enables PGC-1 α to stimulate heme synthesis via transcriptional activation of the rate-limiting enzyme ALAS.

This feedback loop, in addition to dampening Rev-erb α activity, would also tend to reduce the magnitude of fluctuations in heme levels. Our finding that overexpression of Rev-erb α reduces heme levels by $\sim 50\%$ and depletion of Rev-erb α increase intracellular heme about twofold suggest that the Rev-erb α /PGC-1 α cycle functions to keep heme excursions within a fourfold range. The expression of ALAS1 is known to be circadian (Zheng et al. 2001; Kaasik and Lee 2004), regulated by circadian transcription factor NPAS2 (Kaasik and Lee 2004) as well as PGC-1 α (Handschin et al. 2005) and, as shown here, Rev-erb α (via PGC-1 α). Consistent with our model, a recent study measuring heme levels over a 24-h period indeed found that they fluctuate within this fourfold range (Rogers et al. 2008).

It is well established that, at pathologically high levels of heme, HMOX1 is massively induced, resulting in heme breakdown (Ryter et al. 2006). We suggest that the Rev-erb α /PGC-1 α cycle regulates intracellular heme concentration within the physiological range. This negative feedback mechanism would function to help prevent heme concentrations from reaching pathological levels that require detoxification by the HMOX1 system

(Furuyama et al. 2007; Jarmi and Agarwal 2009). In addition, and uniquely, the relief of this negative feedback provides a transcriptional sensing mechanism to stimulate heme biosynthesis when its levels are low. Maintenance of heme sufficiency via the Rev-erb α /PGC-1 α cycle is likely to be critical to normal cellular function. Heme is a prosthetic group for critical enzymes within metabolically active cells, including cytochromes involved in mitochondrial electron transport and oxidative metabolism (Furuyama et al. 2007). It has long been known that heme is essential for the growth and survival of many cell types, and that inhibition of heme synthesis causes cell cycle arrest (Ebert et al. 1979; Tschudy et al. 1980). We showed here that manipulation of Rev-erb α levels alters endogenous heme levels, in a manner that is opposite the change in Rev-erb α , as would be expected for loss of the heme feedback-sensing mechanism. The repression of Rev-erb α on heme biosynthesis indeed affected mitochondrial respiration in a manner that is partly reversed by heme. Moreover, excess Rev-erb α activity is deleterious to cell growth, due to a G2/M cell cycle arrest.

Figure 6. Ectopic Rev-erb α causes cell cycle arrest and blocks cell growth. (A) Ectopic Rev-erb α blocks cell growth. Cell number experiments are expressed as mean $\pm$ SD ($n = 3$). (*) $P < 0.05$. (B) The depletion of Rev-erb α promotes cell growth. Cell numbers are expressed as mean $\pm$ SD ($n = 3$). (*) $P < 0.05$. (C) Ectopic Rev-erb α arrests cells in G2/M phase. The DNA content of NIH3T3 cells was determined by FACS. The percentage of cells in each cell cycle phase was calculated by ModFit Software and is presented in the figure. (*) $P < 0.05$.

Figure 7. The Rev-erb α /PGC-1 α pathway regulating heme homeostasis. Heme promotes Rev-erb α repression of PGC-1 α , thereby reducing ALAS1 gene expression and heme biosynthesis. Conversely, low heme levels reduce Rev-erb α repression, enhancing PGC-1 α stimulation of heme synthesis via transcriptional activation of the rate-limiting enzyme ALAS1.

PGC-1 α has been implicated in the pathogenesis of insulin resistance, neurodegeneration, and other disorders associated with impaired mitochondrial function (Mootha et al. 2003; Patti et al. 2003; Lin et al. 2005; Handschin and Spiegelman 2006; Lin 2009). The link discovered here between heme, Rev-erb α , and PGC-1 α reveals a molecular pathway coordinating and maintaining cellular energy homeostasis, which may be defective or challenged in pathological states such as obesity, diabetes, and cancer. As an NR regulated by reversible binding of a lipophilic heme ligand (Yin et al. 2007), Rev-erb α may be an excellent target for intervention. Indeed, the recent identification of a nonheme, pharmacological ligand for Rev-erb α (Meng et al. 2008) suggests that novel therapies may emerge from the manipulation of Rev-erb α activity.

Materials and methods

Plasmids and reagents

The PGC-1 α luciferase reporter construct was generated by PCR amplification of the 401-bp human PGC-1 α Intron1, which was subcloned into the PGL3 promoter vector using both Kpn1 and NheI sites (Promega). RORE mutants were generated by site-directed mutagenesis using the QuikChange kit (Stratagene) and confirmed by sequencing analysis. The expression vectors encoding human Rev-erb α have been described previously (Yin and Lazar 2005). Protein A-Sepharose was obtained from Amersham Biosciences. Heme and succinylacetone were purchased from Sigma.

Mammalian cell culture and transfection

HepG2 and HEK-293T cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). NIH3T3 cells were maintained in high-glucose DMEM supplemented with 10% bovine serum. NIH3T3 cells stably transfected with stabilized Rev-erb α (S55D/S59D) under the negative control of doxycycline have been described previously (Yin et al. 2006).

Cells were grown at 37°C in 5% CO₂. All transient transfection assays were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For repression assays, cells were grown in 24-well plates and transfected with 0.1 μ g of PGC-1 α luciferase reporter, 0.1–2 μ g of Rev-erb α expression vector, and 0.1 μ g of β -galactosidase expression vector. The total amount of expression plasmid transfected per well was kept constant by adding varying amounts of empty vector. At 48 h posttransfection, cells were lysed and their luciferase activity was assayed using a reporter assay kit (Promega). Luciferase units were normalized to β -galactosidase expression. Fold repression was calculated as the activity of the same reporter in the presence of Rev-erb α expression vector, with the control group (PCDNA3.1) normalized to 1. Each experiment was performed three times in triplicate. In some experiments, succinylacetone was added for 16 h before cell harvesting, and heme was added at various concentrations and times as described (Yin et al. 2007).

Animals and administration of recombinant AAV

All mouse studies were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Eight-week-old male C57BL/6 mice were purchased from Jackson Laboratories and maintained in 12-h light/dark conditions for 4 wk before experiments. Mice were given unrestricted access to water and maintained on a normal chow diet. AAV viral particles were injected via tail vein at a dose of 1×10^{12} genome copies per mouse. Mice were randomly divided into three groups and injected with AAV-GFP and AAV-Rev-erb α SD55/59. At time of necropsy, the livers were harvested for RNA analysis.

Isolation of primary mouse hepatocytes

Primary hepatocytes were isolated from 3-mo-old male C57BL/6 mice. Briefly, following anesthetization with Avertin (1.0 g/kg i.p.; Sigma), the vena cava was cannulated and the liver was perfused with 37°C Liver Perfusion Buffer (Gibco) for 5 min. Next, the perfusion buffer was replaced with 37°C Liver Digestion Buffer (Gibco), supplemented with collagenase 1 (0.2% final concentration; Sigma) for 10 min. Following digestion, the liver was removed and cells were dispersed in DMEM containing 10% FBS and 1% pen per strep. Cells were filtered through a 100- μ m mesh, pelleted, and subjected to a Percoll gradient (45% Percoll in DMEM) to separate live and dead cells. Following three washes in DMEM, viable cells were plated at a density of 0.3×10^6 cells per well in 12-well plates coated with collagen 1 (BD BioCoat) in DMEM, 10% FBS, and 1% pen per strep. Five hours later, unattached cells were removed by washing with DMEM. Cells were washed three times with warm phosphate-buffered saline to remove glucose and cultured in the glucose-free medium containing gluconeogenic substrates (20 mM sodium lactate, 2 mM sodium pyruvate). Cells were stimulated with dexamethasone (1 nM) and 8-CPT-cAMP (500 μ M) with or without heme.

Transduction of HepG2 cells by adenovirus infection

HepG2 cells (3×10^5) were infected with adenovirus expressing control, shRev-erb α , or shPGC-1 α (gift of J. Estall and B. Spiegelman) at a dose of 1×10^8 plaque-forming units.

RNAi

Vectors expressing hairpin siRNAs under the human U6 promoter were constructed by inserting pairs of annealed DNA

oligonucleotides into prelinearized pEntry-U6 vector according to the manufacturer's instruction (Invitrogen Life Technologies). Control was pEntry β -galactosidase plus pSilence Scramble siRNA. The target sequences were as follows: for human Rev-erb α , 5'-GGCATGGGTGTTACTGTGTAATA-3'; for β -galactosidase, 5'-GTGCACCTGGTAAATCTTAT-3'; and for HDAC3, 5'-CAGCGCATTGATGACCAGAGTTACA-3'. Double-strand siRNA oligo for NCoR was purchased from Dharmacon. Cells in 12-well plates were transfected twice over a 96-h period with 1.6 μ g of siRNA vector per well. After the second transfection, cells were harvested for RNA analysis or protein analysis. In the experiment shown in Figure 2B, double-strand RNAi oligos targeting human Rev-erb α were synthesized by Invitrogen. The control sequence was 5'-GACCCUCGUAAGACGCUUCCA AAGU-3'. The Rev-erb α oligo was 5'-ACUUUGGAAGCGUCU UACGAGGGUC-3'.

ChIP assay

Cells were grown in 10-cm plates and either transfected or treated with 6 μ M heme for the indicated experiments. After cross-linking in formaldehyde, cells were lysed in hypotonic buffer (50 mM Tris-HCl, 85 mM KCl, 0.5% Nonidet P-40, 1 $\times$ protease inhibitor). The nuclear fraction was resuspended in 500 μ L of sonication buffer (0.01% SDS, 10 mM EDTA, 50 mM Tris-HCl, 1 $\times$ protease inhibitor) and sonicated four times for 12 sec each followed by centrifugation at 14,000g for 10 min. Supernatants were collected and diluted in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM Tris-HCl, 167 mM NaCl) followed by preclearing with 2 μ g of salmon sperm DNA and protein A-Sepharose for 2 h at 4°C. Immunoprecipitation with the following antibodies was performed overnight at 4°C: anti-acetyl histone H4 (Upstate Biotechnologies), anti-NCoR and anti-HDAC3 (Abcam), and anti-Rev-erb α (Cell Signaling). Immunoprecipitated complexes were collected with protein A-Sepharose beads followed by sequential washes in low-salt, high-salt, lithium, and Tris-EDTA buffers. Precipitates were eluted, and 5 M NaCl was added to reverse cross-links for 6 h at 65°C. DNA fragments were column-purified (Qiagen), and 2 μ L of purified DNA was used in quantitative PCR (QPCR) using primers encompassing both ROSE regions of the human endogenous PGC-1 α Intron1 (forward, 5'-TGTTTGCTGTCATCC TAAAACG-3'; reverse, 5'-TGGGGTGTATGTCATGTGAA-3'). An irrelevant region (PGC1 α exon 13 coding sequence) worked as a negative control (forward, 5'-GAATTGGCAGGTGGAAA AAA-3'; reverse, 5'-ATGTGAAGTCTGATTTGATGG-3').

Quantitative RT-PCR

Total mRNA was prepared using the RNeasy kit (Qiagen). Reverse transcription was performed with 1–2 μ g of total RNA using a reverse transcription kit (AB high-capacity CDNA reverse transcription kit) according to the manufacturer's instructions. The cDNA was subject to quantitative RT-PCR using SYBR Q-PCR mastermix (Applied Biosystems) on a Prism 7900 HT detection system. The primer pairs for amplifying human Bmal1, mouse GAPDH, human PGC-1 α , and mouse PGC-1 α were purchased from Qiagen. Mouse COX5A, mouse IDH3, mouse MCAD, mouse Cytochrome c, and mouse NDUFS8 primer sets were described previously (Cunningham et al. 2007). The sequences for other primer pairs used in this study were human Alas1 (F, 5'-GGCATCCATTAGCATCTGTCTC-3'; R, 5'-GGCTTCATCTTACCACCTC-3'), human glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (F, 5'-GAAGGTGAAGG TCGGAGTC-3'; R, 5'-GAAGATGGTGATGGGATTTC-3'), and

mouse Alas1 (F, 5'-TGCAGAAGGCAGGAAAGTCT-3'; R, 5'-AGGGGTTTCTTTGACCTGCT-3').

Target gene expression was normalized to housekeeping gene GAPDH. The average value from each triplicate was used to calculate fold induction of the gene, with the control group normalized to 1.

Immunoblotting

Cells were lysed in whole-cell lysis buffer (150 mM NaCl, 10 mM Tris at pH 7.6, 0.1% SDS, 5 mM EDTA) with 1 $\times$ protease inhibitor. Twenty micrograms of lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were probed with the following antibodies: anti-NCoR (Ishizuka and Lazar 2003), anti-HDAC3 (Abcam), anti- β -actin (Sigma), anti-GSK3 β (Abcam), anti-PGC1 α (Santa Cruz Biotechnologies), anti-Rev-erb α (Cell Signaling), and anti-Hsp90 (Santa Cruz Biotechnologies).

Heme measurement

Measurement of cellular heme content: Heme in HepG2, NIH3T3 cell lysates was measured by using a modified QuantiChrom Heme Assay (BioAssay Systems) (Raghuram et al. 2007; Rogers et al. 2008). Heme concentrations were normalized to protein. The average heme concentration (micromolar heme per micrograms of total protein) was used to calculate fold changes, with the control group normalized to 1.

Oxygen consumption rate measurement

Cells were seeded at 50,000 cells per well in 24-well XF plates and incubated overnight at 37°C in 5% CO₂ incubator. The oxygen consumption rate is measured by using The Seahorse XF24 Analyzer (Seahorse Bioscience). Immediately before measurement, medium was replaced with nonbuffered pH 7.4 speciality medium. Three successive 10-min measurements were performed simultaneously at 5-min intervals in the triplicates wells. Immediately after measurement, the protein concentration was measured by Bio-Rad protein assay. Oxygen consumption rate was normalized to protein. The cells were measured for the basal oxygen consumption rate and also the oxygen consumption rate after the addition of 5 μ M oligomycin to inhibit ATP synthase. The difference of oxygen consumption rate between the basal and the oligomycin-treated is considered as the oxygen consumption rate used for ATP synthesis, therefore called "respiration-driven oxygen consumption rate" (Watanabe et al. 2006; Wu et al. 2007; Ferrick et al. 2008). The average "respiration-driven oxygen consumption rate" was used to calculate fold changes, with the control group normalized to 1.

FACS analysis

Cells were trypsinized, washed with PBS, and fixed with ice-cold 70% ethanol overnight at 4°C. Nuclear DNA was stained using a solution with 50 μ g/mL propidium iodide (Sigma) and 1 mg/mL RNase A in PBS. The cells were analyzed on FACS Calibur (BD Biosciences) using CellQuest and ModFit data analysis software.

Cell growth curve

To compare the cell growth rate, 1×10^5 to 5×10^5 cells of each cell line were seeded in 12-well plates, and the number of cells of each cell line was counted daily by Vi-CELL Cell Viability Analyzers (Beckman). Each experiment was set up in triplicate

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Negative feedback maintenance of heme homeostasis by its receptor, Rev-erb α

Nan Wu, Lei Yin, Elyisha A. Hanniman, et al.

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