Dietary Iron Controls Circadian Hepatic Glucose Metabolism through Heme Synthesis

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Abstract

The circadian rhythm of the liver maintains glucose homeostasis, and disruption of this rhythm is associated with type 2 diabetes. Feeding is one factor that sets the circadian clock in peripheral tissues, but relatively little is known about the role of specific dietary components in that regard. We assessed the effects of dietary iron on circadian gluconeogenesis. Dietary iron affects circadian glucose metabolism through heme-mediated regulation of the interaction of nuclear receptor subfamily 1 group d member 1 (Rev-Erbα) with its cosuppressor nuclear receptor co-repressor 1 (NCOR). Loss of regulated heme synthesis was achieved by aminolevulinic acid (ALA) treatment of mice or cultured cells, to bypass the rate-limiting enzyme in hepatic heme synthesis, ALA synthase 1 (ALAS1). ALA treatment abolishes differences in hepatic glucose production and in the expression of gluconeogenic enzymes seen with variation of dietary iron. The differences among diets are also lost with inhibition of heme synthesis with isonicotinylhydrazine (INH). Dietary iron modulates levels of peroxisome proliferator-activated receptor gamma, coactivator 1α (PGC-1α), a transcriptional activator of ALAS1, to affect hepatic heme. Treatment of mice with the antioxidant n-acetylcysteine (NAC) diminishes PGC-1α variation observed among the iron diets, suggesting that iron is acting through reactive oxygen species signaling.
Introduction

Circadian rhythms are endogenous cycles of behavioral and physiological processes that are set by external signals called zeitgebers. Light is the zeitgeber for the central clock of the suprachiasmatic nucleus (SCN) that controls circadian movement, feeding behavior, and thermoregulation (1). These SCN driven physiological factors contribute to the synchronization of clocks in peripheral tissues, but peripheral clocks also entrain to non-SCN zeitgebers. Notably, the liver is entrained to food intake and can become dyssynchronous from the SCN (2). Both circulating glucose and hepatic gluconeogenesis are regulated by circadian factors, leading to the hypothesis that dyssynchrony between central and peripheral clocks may contribute to the observed association between metabolic dysregulation and altered sleep rhythms (3).

The precise contribution of specific nutrients to setting the hepatic clock is incompletely understood. One micronutrient, iron, has significant effects on metabolism. Iron affects metabolic regulation through multiple mechanisms including effects on AMP-dependent kinase, fuel preference, insulin secretion, and regulation of the insulin-sensitizing adipokine, adiponectin (3; 4). Iron overload and excess dietary iron are also significant risk factors for diabetes (5-7). Iron is a particularly attractive candidate for contributing to changes in circadian metabolism: Not only is iron an essential component of several proteins involved with electron transport and metabolism, but several circadian transcription factors bind heme. Heme, for example, is necessary for the formation of the complex of nuclear receptor subfamily 1 group d member 1 (Rev-Erbα) with nuclear receptor co-repressor 1 (NCOR), part of the negative arm of the circadian transcriptional feedback loop (8; 9). Heme has also been reported to bind the circadian proteins Clock
and Per2, although the functional relevance of this binding is unknown and has been questioned (10-12).

To test the hypothesis that dietary iron intake may affect circadian metabolic rhythms, we fed mice chow with various iron concentrations, creating tissue iron levels within the range found from normal variation in human diets. We demonstrate that dietary iron content affects gluconeogenesis and circadian rhythm through modifying hepatic heme levels with concordant changes in Rev-Erbα binding to NCOR. Iron elicits these effects through peroxisome proliferator-activated receptor gamma, coactivator 1α (PGC-1α) mediated regulation of the rate-limiting enzyme of heme synthesis in non-erythroid cells, amino levulenic acid synthase 1 (ALAS1).

**Materials and Methods**

*Animal Studies.* Three month old male C57BL6/J mice were fed diets of 35 mg/kg (TD.10211), 500 mg/kg (TD.10212), or 2 g/kg carbonyl iron (TD.10324) (Harlan Teklad Madison, WI by weight 17.7% Protein, 60.1% Carbohydrate, and 7.2% fat) for 6 weeks before *in vivo* physiological testing and 9 weeks before sacrifice. The 35 mg/kg diet is derived from the AIN93G diet, the 500 mg/kg diet is based on animal facility diets which range from 200 to 500 mg/kg, and the 2g/kg diet was selected due to the modest 2-fold increase in liver iron stores, which is within the 4-fold range seen in hepatic iron among humans without iron-related pathology (13). The effects of heme iron were also assessed, heme iron diet was provided to the mice as previously described by Ijssennagger *et al.* (2012)(14).

The animals were maintained on a 12hr light/dark cycle. Treatment of mice with 2mg/mL aminolevulinic acid (ALA) (15) (Frontier Scientific #A167), 1mg/mL
isonicotinylhydrazine (INH) (16)(Sigma Aldrich #I3377), or 6.5mg/mL n-acetylcysteine (NAC) (17) (Sigma Aldrich #A9165) in their drinking water and adjusted to pH 7. Water intake was monitored to ensure there were no variations. ALA treatment began after 6 weeks on diet and continued for 3 weeks concurrent with continued diet. Mice were treated with NAC or INH for 10 days after 8 weeks on diet. In the case of NAC treatment, water was changed every two days to prevent oxidation. Prior to glucose tolerance test (GTT) mice were fasted for 6 hours and then challenged with 1mg glucose per gram body weight through IP injection. For pyruvate tolerance test (PTT), ad libitum fed mice were challenged with 2mg pyruvate per gram body weight through IP injection. To monitor movement, food intake, and respiration mice were housed in the Comprehensive Laboratory Animal Monitoring system for 7 days with the first three days as an acclimatization period (CLAMS; Columbus Instruments, Columbus, OH). For transcriptional studies mice were harvested at 4 hour time points at ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22. Procedures were approved by the Institutional Animal Care and Use Committee of the University of Utah and the Veterans Administration.

**Blood and serum measurements.** Hematocrit and Hemoglobin were measured by Vetscan hm5, while insulin and glucagon were measured by ELISA (EMD Millipore Corp., Billerica, MA, USA; Phoenix Pharmaceuticals Inc., Burlingame, CA, USA).

**Cell Culture.** HepG2 cells (ATCC) were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 supplemented (DMEM/F12) with 10% FBS and .1% primocin. The cells were plated on 6 well plates overnight and then treated with ferric ammonium citrate (FAC,10µM), ALA (100µg/mL), INH (5mM) or a PBS no treatment control for 12 hours. After the drug or iron treatment the plate was synchronized with a
100nM dexamethasone (DXS) shock for one hour, after this time the media was removed the cells washed with PBS and DMEM/F12 with treatment was replaced. Cells were harvested at 4 hour intervals from the time of shock. Knock down of PGC-1α was accomplished by PGC-1α siRNA from Santa Cruz Biotech (sc-38885), following the companies’ general protocol with control siRNA (sc-37007) and transfection reagent (sc-29528).

**RT-PCR.** RNA was isolated from liver samples using RNeasy (Qiagen) and was measured by nanodrop (EPOCH). cDNA was synthesized with the superscript III first-strand synthesis system (Invitrogen). Measurement of relative abundance was performed by real-time PCR analysis using the Power SYBR Green Master Mix (Applied Biosciences) on a QuantStudio™ 12K Flex System with 384-well Block. The standard curve was prepared by serial dilution of pooled cDNA from each sample using five log₁₀ standards. Rpl13 and cyB were selected as reference genes because they do not fluctuate temporally, or vary with dietary iron (data not shown). Quantities were calculated by QuantStudio™ expression suite software based on the standard curve. Primers were designed using the NIH facilitated Mouse Primer Depot (http://mouseprimerdepot.nci.nih.gov/).

**Western blotting.** Antibodies used included Rev-Erbα (Santa Cruz Biotechnologies Inc.), NCOR, β-actin (Cell Signaling), and PGC-1α (Abcam). The Criterion running and transfer system with precast gels was used (BioRad).

**Heme measurement.** Heme was measured by pyridine hemochrome spectrophotometric assay and HPLC (18). In the pyridine hemochrome assay, heme was extracted from tissue samples with 500mM NaOH, pyridine added to 40%. Wave
scans measured from 450 to 620nm using an Ultrospec 3000 spectrophotometer, after addition of 1M potassium ferricyanide and sodium dithionite crystals. Data was analyzed using published extinction coefficients. For reverse phase HPLC measurements, liver samples were homogenized in acidified Acetone, filtered (0.45uM) and HPLC measurements performed on a Waters 2690 pump with C18 reverse phase column (39X300mm Waters µBondapak) and C18 precolumn (Water 2690; Water 996 phospho-diode array detector). Samples (0.125ul) were eluted over a 15 minute period using a linear gradient of 60% buffer A (.56mM NH₄H₂PO₄, pH 7.0) to 100% Methanol.

Iron measurement. Equal amounts of liver lysate were digested with metal free nitric acid for 12 hours at 95˚C (Optima; PerkinElmer, Boston, MA). Samples were centrifuged at 12,000 x g for 20 minutes, and diluted with nanopure water (18megaohm). Metal content was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 3100XL; Perkin Elmer). While Non-heme iron measurements were performed by colorimetric assay (19). Standard curves were made from 1mg Fe/mL solution (Fisher PFE1KN100).

Iron absorption and Transferrin saturation. The iron absorption protocol used was previously described in Ajioka et al. (2002). Mice were fasted overnight and ⁵⁹FeCl₃ was administered (200 µCi/kg body weight) by gavage. After 24 hours the mice were sacrificed, the gastrointestinal tract harvested, and the radioactivity measured using a gamma counter (Perkin Elmer Instruments, Shelton, CT). Transferrin saturation was measured using an iron and iron binding capacity kit (Sigma-Aldrich).

ChIP. ChIP studies were performed as previously described (4) using the Simple ChIP kit with company modifications for tissue samples (Cell Signaling Technology). Liver
samples were homogenized in bead mill tubes with 1.44mm ceramic beads and then cross-linked for 20 minutes with 1% formaldehyde, the crosslinking was stopped with glycine and the lysates were sonicated 3x for 20 seconds using a sonic dismembrator (Fisher Scientific). Lysates were precleared, and A/G agarose beads were blocked with salmon sperm and BSA A/G (Millipore). NCOR, IgG, or Histone H3 antibody (Cell signaling #5948, #2729, #4620) was applied for one hour at room temperature. DNA was released from protein-DNA complexes by proteinase K digestion and quantified by real-time PCR for the enrichment at the PEPCK and G6Pase promoter as well as using GAPDH and the PEPCK gene as negative controls to ensure appropriate shearing using the Power SYBR Green Master Mix (Applied Biosciences)(8). Occupancy was quantified to standard control and normalized to input control.

Coimmunoprecipitation Liver protein lysates isolated in a standard HEPES lysis buffer were pre-cleared with Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnical Inc.). Rev-Erbα antibody (Santa Cruz Biotechnical Inc.) or a negative control IgG (Cell signaling) in 1x PBS was incubated with end over end mixing for 4 hours at 4˚C with Protein A/G PLUS-Agarose beads. The beads were washed 5x with PBSCM (PBS buffer (100 mM sodium phosphate, 150 mM NaCl, pH 7.2) with 1 mM CaCl2 + 1 mM MgCl2). The protein lysate samples were then incubated with the beads overnight. Following protein incubation the beads were washed with PBSCM and then samples were eluted with sample loading buffer.

Metabolite measurement. This procedure was done as previously described (20). Briefly, 20mg of freeze clamped tissue was homogenized in bead mill tubes containing 500µL of cold 90% MeOH containing internal standards. After a 20,000xg centrifugation,
the supernatant was collected and the pellet was extracted again with cold 60% MeOH solution. The supernatants were combined and vacuum dried. Samples were reconstituted with 48 µL of 10 mM sodium phosphate buffer (pH 7.0) followed by 2 µL of 2-vinylpyridine, and after a 30 minutes at room temperature, 50 µL of 20 mM ammonium formate buffer pH 9.2 containing 7.5 mM N-butyl amine was added. After centrifuging at 20,000xg for 5 minutes, 20 µL of sample was injected into Phenomenex (Torrance, CA) Gemini-NX C18 (150x3mm; 3 um particle size, 110A pore size). Data were normalized to weight and internal standard.

Statistics. Statistics were done using JMP pro statistical package using a two-tailed ANOVA with a Tukey-Kramer post hoc analysis to determine differences between multiple groups, these values are listed in the supplemental. Results are presented as the mean ± the standard error of the mean.

Results

*Dietary iron affects circadian glucose metabolism.*

C57BL/6J male mice were fed high (H, 2g iron/kg chow), high normal (HN, 500mg iron/kg chow), or low normal (LN, 35mg iron/kg chow) iron diets for 6 weeks. These levels of dietary iron are not so extreme as to cause neuropathy and allowed for normal hemoglobin concentrations and red blood cell volumes (hematocrit) to be maintained, but resulted in differences in blood transferrin saturation and gut iron absorption (Table 1,(21)). In the liver increasing dietary iron led to significant increases in non-heme iron and total iron, decreased transferrin receptor, and increased hepcidin transcripts (Table 1, Figure S1A-D). These changes are consistent with our previously published observation that liver ferritin protein levels increase in high iron diets (20).
Changes in dietary iron also resulted in differences in glucose homeostasis. The area under the glucose curve (AUCg) during GTT significantly varied with dietary iron at zeitgeber time 0 (ZT0, lights on) and ZT12 (lights off). Within diets, the AUCg varied significantly with time of day, as has been reported previously (22)(Figure 1A). Fasting glucose values also differed among the dietary groups at ZT18 (Figure 1B). To elucidate the mechanism behind these differences in glucose we first measured ad libitum circulating insulin levels and saw no variation that would explain the changes (Table 1, Figure S2A). We next assessed gluconeogenesis because it is a major contributor to serum glucose concentrations, and its dysregulation is seen early in diabetes. We have previously reported that high dietary iron significantly decreases gluconeogenesis as measured by using [U-13C6]-D-glucose (20). We validated these data by PTT performed at ZT12 when mice normally begin feeding and down regulate gluconeogenesis (Figure 1C and D). Mice fed the LN diet exhibited higher levels of blood glucose in response to pyruvate injection compared to mice on the HN or H diets. Variations in glucagon did not account for these differences (Table 1). The LN-fed mice weighed 1.8 and 1.2 g more than the HN- and H-fed mice, respectively, although weight and AUCg were not significantly correlated across all individuals (Table 1; p=.3944, not shown).

We next assessed the transcript levels of gluconeogenic enzyme in the livers of the mice on the different iron diets. Consistent with the AUCg after pyruvate tolerance testing, the mRNA levels of PEPCK exhibited a significantly greater peak in mice on the LN diet when compared to the H- and HN-fed mice (Figure 1E). Glucose-6-phosphatase (G6Pase) peak levels trended higher in the LN compared to the H group (Figure 1F). Feeding behavior did not differ significantly (Figure S2B).
Iron alters Rev-Erbα repressor complex formation and activity

Because the parameters of glucose homeostasis exhibited temporal fluctuations that were dependent on dietary iron content, we next examined circadian regulatory factors that are associated with iron. Rev-Erbα is a member of the circadian regulon that directly inhibits PEPCK and G6Pase expression. This repressive activity requires heme for Rev-Erbα to bind NCOR to form the repressor complex (8). We assessed complex formation at ZT14, a time when circadian repression of gluconeogenic enzyme expression is beginning to manifest, by performing an immunoprecipitation of Rev-Erbα followed by immunoblotting for NCOR. We observed a direct correlation of complex formation with increased dietary iron (Figure 2A and B), consistent with the observed decreases in gluconeogenesis and levels of gluconeogenic enzyme transcripts with higher dietary iron. Complex formation correlated with occupancy at ZT14 of the PEPCK and G6Pase promoters by NCOR, as revealed by chromatin immunoprecipitation (ChIP) (Figure 2C & D). We did not observe variations in NCOR and Rev-Erbα transcript or protein between the diets at ZT14 (Figure S3A-F).

Dietary iron alters heme levels and temporal expression of enzymes involved in heme synthesis and degradation

The heme dependence of Rev-Erbα/NCOR repressor complex formation prompted us to measure hepatic heme levels. Livers were perfused with PBS to remove red blood cells, and total heme levels were assessed by HPLC (Figure 3A and Figure S4A). A pyridine hemochromagen assay was performed to determine levels of heme B, the form that binds Rev-Erbα (Figure 3B and Figure S4B) (9). In both assays heme was highest
in the H fed mice at ZT12, which was the time at which we observed a rise in Rev-
Erβα/NCOR complex formation (Figure 2A and B). Conversely, at ZT0 heme levels
were highest in the LN fed mice (Figure S4A and B). The net result of these changes is
that heme B levels show higher temporal variation in mice fed higher iron diets, as
reflected in the ZT12/ZT0 ratios (LN=.778, HN=1.69, and H=3.02). Peak transcript
levels of ALAS1 (Figure 3C) increase with increased heme at ZT10, and heme
oxygenase 1 (HO-1), an enzyme necessary for heme catabolism, showed a similar
trend with dietary iron at ZT18 (Figure S4C). When mice were fed high dietary heme we
also saw similar results in PTT, gluconeogenic enzyme transcripts and in heme levels
(Figure S5A-D).

If ALAS-1-mediated changes in heme levels are driving the changes in Rev-Erβα/NCOR
association, we hypothesized that bypassing ALAS-1 should increase heme levels and
abrogate the effects of dietary iron on gluconeogenesis. Inhibiting heme synthesis
should decrease heme levels, likewise limiting the effects of dietary iron. Treatment of
mice with the product of ALAS1, ALA has been shown to increase heme levels in the
liver and erythrocytes (15). Mice were therefore fed the LN, HN, and H iron diets for six
weeks and concurrently treated with 2mg/mL ALA in their drinking water for the final 3
weeks. At the end of this period, we determined heme levels in perfused liver by HPLC.
Heme levels increased in all three diets (Figure 4A). Heme B levels showed a similar
trend (Figure S4D)). We performed PTTs on the ALA treated and control mice at ZT12;
ALA treatment abolished differences between the LN-, HN-, and H-fed mice (Figure 4B).
ALA treatment also diminished the differences in PEPCK and G6Pase mRNA levels
observed in the control mice with the various iron diets (Figure 4C and D). In general,
the effect of ALA treatment was to decrease the PTT-AUCg and transcript levels in the LN mice toward the levels seen in the mice on the higher iron diets. Furthermore, IP of Rev-Erbα in ALA treated and control livers showed increased NCOR coprecipitation with ALA treatment (Figure 4E and F).

INH inhibits heme synthesis by decreasing pyridoxine, a cofactor for ALAS1. Mice were fed the iron diets for 8 weeks and then treated with INH in their drinking water for 10 days while continuing the iron diets. PTT was performed after five days of INH treatment, and tissues were harvested after 10 days. The results were the converse of those observed on ALA: The effect of INH was to increase the lower AUCg seen in the HN and H diets to a level resembling that observed in the mice on LN diet (Figure 4H). Similar effects were noted for PEPCK and G6Pase transcript levels (Figure 4I and 4J). The INH treatment also decreased Rev-Erbα/NCOR complex formation in all of the iron diets, with the greatest relative effect seen in the H group (Figure 4K and 4L).

Dietary iron alters gluconeogenesis cell-autonomously in a human hepatoma model

To determine if dietary iron affects hepatocyte glucose production in a cell-autonomous manner as opposed to exerting more indirect (e.g. hormonal) effects, we treated HepG2 cells with FAC. Cells were grown for twelve hours in control DMEM or DMEM with 10µM FAC, with or without treatment of ALA or INH. We then set a circadian rhythm in the cells with DXS shock (23). The results were concordant with in vivo results. At the peak expression of PEPCK and G6Pase, non-iron-treated cells exhibited a greater PEPCK and G6Pase expression compared to the FAC treated cells (Figure 5A and B). Treatment of cells with the iron chelator deferoxamine ablated these rhythms in PEPCK and G6Pase (Figure S6A and B). ALA treatment brings PEPCK and G6Pase transcript
levels down in non-iron-treated cells, close to those observed in the FAC-treated cells (Figure 5A and B, right panels). Conversely, INH increases transcript levels in the FAC treated cells, close to those observed in non-iron-treated cells (Figure 5A and B, left panels). During the peak times we also assessed NCOR association with Rev-Erbα through a coprecipitation and were able to recapitulate the trends observed in the animals: FAC treatment increased association of NCOR and Rev-Erbα (Figure 5C and D, middle panel). ALA treatment increased complex formation to levels seen in the FAC-treated cells, while INH treatment decreased association to levels seen in the non-iron-treated cells (Figure 5C and D, left and right panels respectively).

Variations in heme synthesis are due to differences in PGC-1α expression related to altered oxidative signaling in mice fed the various iron diets

PGC-1α regulates ALAS1 transcription (24), leading us to explore its possible regulation by iron to explain the differences in heme synthesis between the iron diets. In mice, both PGC-1α transcript and protein levels increased with increasing dietary iron (Figure 6A-C). In HepG2 cells, partial silencing of PGC-1α by siRNA treatment (Figure 6D) ablated the differences in ALAS1, PEPCK, and G6Pase that occurred with FAC treatment (Figure 6E, F, and G).

PGC-1α is up regulated by reactive oxygen species (ROS) (25; 26), and iron is known to create ROS through fenton chemistry. Therefore we explored oxidative signaling as a mechanism through which dietary iron up regulates PGC-1α. Cellular oxidative markers
differed temporally among the diets, with lower GSH levels (Figure 7A) and NADPH/NADP+ ratios (Figure 7B) in the H fed mice, as well as the transcripts of enzymes involved in the oxidative response such as superoxide dismutase 1 (Figure S7A and B). To blunt differences in oxidant levels among mice on the iron diets we treated them with the antioxidant N-acetylcysteine (NAC). Differences observed in fasting glucose observed at ZT12 between the mice on LN, HN, and H diet were reversed by NAC treatment: NAC had no effect on fasting glucose in the LN mice but increased glucose in mice on the HN diet and even more so in mice on the H diet. The livers of the mice were harvested at ZT12. NAC treatment abolished differences in PGC-1α, PEPCK, G6Pase, ALAS1, GSH, and NADPH/NADP+ ratios between the diets (Figure 7D-I).

**Discussion**

Iron is a risk factor for several parameters of metabolic syndrome. For example, a high serum ferritin level, a marker of tissue iron stores, is associated with insulin resistance and type 2 diabetes (27). This relationship between iron and metabolic syndrome highlights the role of iron as both a structural component of proteins involved in bioenergetics, and as a cellular regulator of metabolic pathways such as AMPK activity (4; 20). We show herein yet another role of iron in metabolism, namely that dietary iron affects the circadian rhythm of hepatic gluconeogenesis by altering heme synthesis that in turn affects the activity of a key component of the circadian machinery, Rev-Erbα (Figure 8).
Besides being a crucial component of the circadian clock, Rev-Erbα regulates many aspects of glucose and lipid metabolism, including acting as a negative transcriptional regulator of gluconeogenic enzymes. Decreased Rev-Erbα expression is associated with hyperglycemia and obesity in mice and humans (8; 28-30). Previous work has shown that Rev-Erbα activity is dependent on the formation of a repressor complex with NCOR, which requires heme (8). We verified the dependence of this complex formation on heme availability, and further showed that liver heme levels temporally fluctuate and vary with dietary iron. The results demonstrate that heme availability is altered by dietary iron and is a regulating factor in the circadian rhythm of hepatic gluconeogenesis. While heme is known to be required for Rev-Erbα/NCOR complex formation, the observations that heme availability is limiting for that process in normal physiologic circumstances has not been previously shown.

The changes in heme levels observed with increased dietary iron are not due to limiting amounts of iron for metalation of heme but rather by the regulation of heme synthesis by ALAS1. This is revealed by the facts that bypassing ALAS1 with ALA, or inhibiting heme synthesis by INH abrogated the effects of dietary iron. If iron itself were limiting in the LN diet, for example, ALA would not be able to increase heme-dependent formation of the Rev-Erbα/NCOR complex in mice on that diet. Furthermore, mice on the lower levels of dietary iron show no evidence of systemic iron deficiency (Table 1). These data are supported by recent studies in humans which show that treatment with ALA is able to reduce fasting blood glucose (31).

ALAS1 itself exhibits a circadian rhythm controlled by PGC-1α and its binding partners NRF-1 or FOXO1 (32). We confirmed the circadian rhythm of ALAS-1 transcripts in the
current study and show that the magnitude of its excursions is significantly altered by dietary iron. The regulation of ALAS-1 is not mediated directly by iron responsive proteins, as it is for the erythroid form of the enzyme ALAS2 (33). Rather, iron drives increased PGC-1α expression that in turn transcriptionally activates ALAS1. This was demonstrated by the fact that the effects of iron on ALAS1, PEPCK, and G6Pase were not seen after knockdown of PGC-1α in HepG2 cells. The circadian timing of these events in vivo corresponds to the feeding behavior of mice which is crepuscular in a laboratory setting with peaks in food intake seen ZT0 and ZT12. It is the food intake at ZT0 that is able to modulate the oxidative setting and synchronize the later peaks in heme synthesis.

PGC-1α is a transcriptional coactivator that is known to be involved in a number of diverse metabolic pathways including circadian rhythms, mitochondrial biogenesis, adaptive thermogenesis, and metabolism of both glucose and lipids (34; 35). Dietary iron has been shown to alter the expression of PGC-1α: Decreased protein levels are observed in the skeletal muscle of rats fed an iron deficient diet, although the mechanism of this change was not determined (36). We demonstrate that iron regulation of PGC-1α occurs at least in part through oxidant sensing pathways. Mice on the higher iron diets show evidence of changes in intracellular redox state as revealed by decreased GSH and decreased NADPH/NADP+ ratios. Treatment of mice with the antioxidant N-acetyl cysteine eliminated the variation seen in PGC-1α, ALAS1, PEPCK, and G6Pase among the different iron diets. These data suggest that dietary iron is acting through ROS to exert effects upon heme synthesis and subsequently gluconeogenesis.
While we have observed significant effects of iron on the expression of gluconeogenic enzymes, the overall regulation of gluconeogenesis is controlled by numerous other hormonal, signaling and transcription factors. Further complicating this intricate pathway is the fact that PGC-1α also regulates gluconeogenesis more directly, increasing transcription of PEPCK and G6Pase through coactivation of hepatic nuclear factor-4α (Hnf4α), glucocorticoid receptor (GR), and forkhead box1 (FOXO1) (37). Thus, the apparently paradoxical observation that PGC-1α increases in H fed mice, while PEPCK and G6Pase transcripts are decreased could be due to multiple promoter inputs from both AMPK and Rev-Erbα/NCOR, or differences in PGC-1α binding partners.

Furthermore, in previous studies we have shown that iron alters FOXO-1-dependent transcription and levels of the FOXO-1 acetylation (4). This system is also complicated by research that shows gluconeogenic signals regulate iron uptake, suggesting that iron metabolism and glucose homeostasis are tightly coupled processes (38).

Previous work on iron and metabolism has concentrated on pathologic iron overload. However, increased diabetes risk is seen even with modest levels of iron excess such as higher dietary iron intake (6; 39). On the surface this correlation of high iron with increased diabetes risk appears contradictory to our results of high iron being associated with improved glucose tolerance and hepatic glucose production. However, type 2 diabetes is a multifactorial disease and the prodiabetic effects of iron on insulin production and adipokines may overtime overwhelm other apparently antidiabetic effects such as those observed on hepatic gluconeogenesis (4; 20; 40). Furthermore, the increased magnitude of circadian variation in hepatic gluconeogenesis seen in the high iron fed mice could be prodiabetic under certain circumstances. For example, in
situations of nightshift work, sleep disturbance, or snacking during the night, all of which are associated with increased diabetes risk (41), it might be expected that individuals with higher iron would suffer greater dyssynchrony and disruption of normal metabolic regulation.

Besides its effects on ROS and heme availability, the effects of iron on metabolism are likely to be widespread and not explained by a single mechanism. The drosophila Rev-Erbα homologue E75 is regulated by nitric oxide (NO) (42), and transcript levels of nitric oxide synthase, the rate limiting enzyme in NO synthesis, were increased with increasing dietary iron at certain time points (not shown). Further, the redox state of thiols within the heme binding pocket of proteins such as Rev-Erbα also regulates heme binding (43).

Although our work has focused on hepatic gluconeogenesis in mammals, the effects of iron on circadian rhythms are also seen in plants (44) and insects (45), and they are manifest in multiple organ systems and tissues including the central clock of the SCN. Iron deficiency in mice, for example, alters thermoregulation, behavior, and monoamine metabolism and dopamine transporter expression in the central nervous system (46; 47). Furthermore, knock down of the drosophila homologue of ferritin in circadian neurons disrupts the molecular components of the central clock (45).

In summary, we have demonstrated that one mechanism through which dietary iron is able to regulate the core molecular clock in the liver is through regulation of heme synthesis. This novel role of iron provides another mechanism through which iron acts
to regulate glucose metabolism. The studies suggest mechanisms that may underlie the interplay among iron, altered circadian rhythms, metabolic regulation, and diabetes risk.

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**List of Acronyms**

Aminolevulinic acid synthase 1 (ALAS1); Aminolevulinic acid (ALA); Area under the glucose curve (AUCg); Ferric ammonium citrate (FAC); Glucose-6-phosphate (G6Pase); Glucose tolerance test (GTT); High iron diet (H); High normal iron diet (HN); Heme oxygenase 1 (HO-1); isonicotinylhydrazine (INH); Low normal iron diet (LN); N-acetylcysteine (NAC); Nuclear receptor co-repressor (NCOR); Nuclear receptor subfamily 1 group d member 1 (Rev-Erbα); Peroxisome proliferator-activated receptor gamma, coactivator 1α (PGC-1α); Pyruvate tolerance test (PTT); Suprachiasmatic nucleus (SCN); Zeitgeber time (ZT)

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Figure legends

**Figure 1. Dietary iron decreases parameters of glucose homeostasis and gluconeogenic transcription**

A) Area under the glucose curve (AUCg) for GTTs performed at various time points after a 6 hour fast (n=10-16). B) Fasting blood glucose levels obtained after 6 hour fast throughout circade (n=10-16). C) *ad libitum* PTT and D) the corresponding AUCg at ZT12 (n=11-16). E) Liver PEPCK and F) G6Pase mRNA (n=6) as determined by RT-PCR normalized to cyB and RPL13, housekeeping transcript which did not display circadian variation (n=6). *p<.05, **p<.01, and ***p<.001.

**Figure 2. Dietary iron affects the formation of the Rev-Erbα repressor complex and its activity**

A) Western blot of Rev-erbα IP extracts blotted for NCOR and Rev-Erbα at ZT14 and B) quantification of these blots (n=6). C) ChIP of NCOR to the PEPCK (n=4) and D) G6Pase promoter determined by RT-PCR normalized to input at ZT14 (n=4). Values are represented as % input which is calculated by dividing the CT value of the DNA recovered from each experimental IP by the CT of adjusted input (the initial input was 2%) and multiplying by 100. IgG control was subtracted out as baseline. *p<.05, **p<.01, and ***p<.001.

**Figure 3. Hepatic heme synthesis and steady-state levels are altered with dietary iron**

A) Total heme levels as measured by HPLC at ZT12 (n=6). B) Heme B levels as measured by hemochromagen pyridine assay at ZT12 (n=12). C) ALAS1 transcripts determined by RT-PCR normalized to cyB and RPL13 (n=6). *p<.05, **p<.01, and ***p<.001.
Figure 4. Drug induced increase of heme ablates variations in heme, Rev-Erbα/NCOR complex formation, and gluconeogenesis observed between the various diets. A) Heme levels as measured by HPLC with ALA treatment and control (n=6-12) B) AUCg from ZT12 PTT (n=6) C) PEPCK (n=9), and D) G6pase mRNA transcripts in ALA treated and control mice normalized to RPL13 and cyB(n=9). E) Western blot of Rev-Erbα IP extracts blotted for NCOR and Rev-Erbα and F) quantification of the density of the NCOR blot normalized to the Rev-Erbα density (n=4-6). G) Heme levels as measured by HPLC with INH treated and control mice (n=3). H) AUCg for ZT12 PTT (n=5-8) I) PEPCK (n=5-8) and J) G6Pase transcript levels as measured by RT PCR normalized to RPL13 and cyclophilin in INH treated and control (n=5-8). K) Western blot for Rev-Erbα/NCOR coIP extracts blotted for Rev-Erbα and NCOR (n=6) and L) quantified density of NCOR bands normalized to Rev-Erbα density (n=6).

Figure 5. Treatment of HepG2 cells with FAC, ALA, or INH recapitulates mouse results. A) PEPCK (n=12-24) and B) G6Pase transcript levels as measured by RT PCR for control and FAC cells with no drug treatment, ALA, or INH treated (n=12-24). C) Western blot of Rev-Erbα IP cell extract blotted for NCOR and Rev-Erbα and D) quantification of the density of the NCOR band as normalized to Rev-Erbα (n=3). *p<.05, **p<.01, and ***p<.001.

Figure 6. Increased Dietary iron increases PGC-1α and knock down of PGC-1α abrogates differences between iron treated and nontreated HepG2 cells. A) PGC-1α mRNA as measured by RT PCR (n=6, ZT6 p=.036, LN vs H p=.0272, ZT10 p=.0495, LN vs H p=.0472). B) Western blot of liver extracts blotted for PGC-1α at ZT10 and C)
Density of PGC-1α band normalized to β actin (n=6, p=.0092, LN vs H p=.0079, HN vs H p=.0734). D) Verification of PGC-1α KD by RT PCR (n=6, Control vs FAC p=.0167) and E) PEPCK, (n=6, Control vs FAC p=.048) F) G6pase (n=6, Control vs FAC p=.037), and G) ALAS1 transcript levels in PGC-1α KD normalized to Rpl13 (n=6).

*p<.05, **p<.01, and ***p<.001.

**Figure 7. Dietary iron alters hepatocyte oxidant signaling and antioxidant treatment ablates differences in PGC-1α and gluconeogenic transcripts**

A) GC MS/MS measurement of GSH (n=6) and B) NADPH/NADP+ in freeze clamped liver of mice on iron diets at various harvest times (n=6). C) Fasting blood glucose measurements in control and NAC treated mice (n=5-10). D) Transcript levels of PGC-1α (n=3-6), E) PEPCK (n=3-6), F) G6Pase (n=3-6), and G) ALAS1 in NAC treated and control mice (n=3-6). H) GC MS/MS measurement of GSH (n=3-6) and I) NADPH/NADP+ in NAC treated or control mice at ZT12 (n=3-6). †p=.147, *p<.05, **p<.01, and ***p<.001.

**Figure 8. Schematic representation of the effects of dietary iron on circadian gluconeogenesis.** Dietary iron alters the oxidative state of the cell to increase the transcription of PGC-1α, which then regulates heme synthesis. These changes in heme synthesis in turn increase the complex formation of Rev-Erbα and NCOR to inhibit the transcription of gluconeogenic enzymes PEPCK and G6Pase to decrease hepatic glucose production.
Table 1

<table>
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<th>Variables</th>
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<th>HN</th>
<th>H</th>
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<td>Hemoglobin (g/dL)</td>
<td>16.4± 0.5</td>
<td>15.6 ± 1.4</td>
<td>16.7 ± 1.7</td>
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<td>Hematocrit (%)</td>
<td>47.9 ± 1.6</td>
<td>45.3 ± 3.4</td>
<td>47.6 ± 4.0</td>
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<td>Insulin (ng/mL)</td>
<td>1.73 ± 0.65</td>
<td>1.52 ± 0.45</td>
<td>2.15 ± 0.38</td>
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<td>Glucagon (pg/mL)</td>
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<td>60.4 ± 5.5</td>
<td>78.1 ± 8.7</td>
</tr>
<tr>
<td>Body weight ‡(g)</td>
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<td>28.09 ± 0.25</td>
<td>28.65 ± 0.176</td>
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<tr>
<td>Total liver iron§(μg/g wet weight)</td>
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<td>239.4 ± 42.5</td>
<td>583.3 ± 79.3**</td>
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<td>Transferin Saturation (%)</td>
<td>44.1 ± 4.1</td>
<td>45.6 ± 4.9</td>
<td>58.5 ± 3.1*</td>
</tr>
<tr>
<td>Available ⁵⁹Fe Absorption (%)</td>
<td>41.3 ± 4.2**</td>
<td>22.4 ± 3.1</td>
<td>8.8 ± 1.5*</td>
</tr>
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Table 1 Physiologic parameters in mice fed diets containing different levels of iron. *p≤.05, **p≤.01, and ***p≤.001 by two-tailed ANOVA with Tukey post-hoc analysis compared to HN.

‡N=72/group

§Measured by ICP MS
Figure 1. Dietary iron decreases parameters of glucose homeostasis and gluconeogenic transcriptio
279x361mm (300 x 300 DPI)
Figure 2. Dietary iron affects the formation of the Rev-Erbα repressor complex and its activity.
Figure 3. Hepatic heme synthesis and steady-state levels are altered with dietary iron

183x200mm (300 x 300 DPI)
Figure 4. Drug induced increase of heme ablates variations in heme, Rev-Erbα/NCOR complex formation, and gluconeogenesis observed between the various diets.

472x586mm (300 x 300 DPI)
Figure 5. Treatment of HepG2 cells with FAC, ALA, or INH recapitulates mouse results.
Figure 6. Increased Dietary iron increases PGC-1α and knock down of PGC-1α abrogates differences between iron treated and nontreated HepG2 cells.

247x146mm (300 x 300 DPI)
Figure 7. Dietary iron alters hepatocyte oxidant signaling and antioxidant treatment ablates differences in PGC-1α and gluconeogenic transcripts.
Figure 8: Schematic representation of the effects of dietary iron on circadian gluconeogenesis
204x185mm (96 x 96 DPI)
Figure S1; related to Table 1. Iron parameters in mice fed the different iron diets, measured at ZT10. A) Non-heme iron measurement in the liver (n=8); transcript levels of B) transferrin receptor 1 (n=6), C) hepcidin at ZT14 (n=6), and D) ferritin (n=6). (*p<.05 compared to HN or 350mg/kg diet).

Figure S2; related to Figure 1. Circadian insulin and food intake in mice fed the different iron diets. A) *Ad libitum* serum insulin (n=3-6, *p=.05, LN vs HN p=.04, LN vs H p=.04, HN vs H p=.77). B) Feeding behavior as measured by electronic scale in the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH; N=6-8).
Figure S3; related to Figure 2. Assessment of Rev-Erbα and NCOR abundance at ZT14. 
A) Rev-Erbα blot B) quantification (n=4, ZT14) C) NCOR blot and quantification. C) 
NCOR blot D) quantification (n=5). Transcripts of E) Rev-Erbα and F) NCOR (n=5-6)
Figure S4; related to Figure 3. Hepatic iron and heme oxygenase mRNA in mice fed the different iron diets. A) ZT0 heme measured by HPLC (p=.3236); B) ZT0 heme B as measured by Hemochromogen pyridine (p=.6361); C) Hmox-1 transcript levels as normalized by RPL13 and cyclophilin B (ZT10 p=.0289, LN vs HN p=.3219, LN vs H p=.2193, HN vs H p=.0137; ZT14 p=.0018, LN vs HN p=.00237, LN vs H p=.0021, HN vs H p=.537; ZT18 p<.0001, LN vs HN p=.0031, LN vs H p=.0002, HN vs H p=.0012). Heme B in D) ALA (n=5-8) and E) INH treated groups vs control (n=4-9)
Figure S5; related to Figure 5. Regulation of hepatic gluconeogenesis and heme at ZT12 with .05μM hemin supplement to the LN diet A) AUCg for a PTT on mice fed LN or LN+Hemin for 6 weeks (n=6, p=.013). B) Heme B as measured by pyridine hemochromagen assay (n=5-6, p=.021). C) PEPCK mRNA (n=6, p<.001) and D) G6Pase mRNA normalized to RPL13 and CyB (n=6, p<.001). *p<.05, **p<.01, and ***p<.001

Figure S6; related to Figure 5. Circadian expression of gluconeogenic genes in HepG2 cells. A) Circadian expression of PEPCK and B) G6Pase in DXS shocked HepG2 cells (n=18) in control, FAC, and deferoxamine.
Figure S7; related to Figure 7 Circadian expression of hepatic transcripts sensitive to oxidative stress in mice fed LN, HN, and H iron diet A) Superoxide dismutase 1 (SOD1) and B) Catalase (n=6)(*p<.05, **p<.01, and ***p<.001)

Tukey-Kramer post hoc analysis for figures:

**Table 1 Physiologic factors of metabolism and iron.** Body weight is measured as n=72 per group with *p<.0001 (LN vs HN p<.0001, LN vs H p=.0019 by Tukey-Kramer posthoc analysis). Iron was measured by ICP MS n=12 per group with *p<.0001 (LN vs HN p=.0445, LN vs H p<.0001, HN vs H p=.0003). Serum transferrin saturation n=5 with *p<.01 (LN vs HN p=.0063, HN vs H p=.045). Fe absorption n=4 *p<.05 (HN vs H p=.021)

**Figure 1 Dietary iron decreases parameters of glucose homeostasis and gluconeogenic transcription** A) Area under the glucose curve (AUCg) for GTTs performed at various time points after a 6 hour fast (n=10-16, ZT0*p=.04, HN vs LN p=.89, LN vs H p=.04, HN vs H p=.04, ZT12 *p=.001, LN vs HN p=.92, LN vs H p=.001, HN vs H p=.001). B) Fasting blood glucose levels obtained after 6 hour fast throughout circade (n=10-16, *p=.0428, LN vs H p=.1405, HN vs H p=.0449). C) ad libitum PTT at ZT12 (30minute p=.0014, LN vs H p=.0016, LN vs HN p=.0169, HN vs H p=.6389; 60 minute p<.0001, LN vs H p=.0002, LN vs HN p=.0002; HN vs H p=.9963 by Tukey-Kramer posthoc analysis)and the D) AUCg (n=12-16, p=.0004, LN vs H p=.001, LN vs HN p=.0021, HN vs H p=.9564) E) Liver PEPCK (n=6, ZT0 p=.0257 LN vs HN p=.0261, LN vs H p=.7782, HN vs H p=.0939; ZT6 p<.0001, LN vs HN p=.0001, LN vs H p=.0002, HN vs H=.9992; ZT10 p=.0192, LN vs HN p=.0237, LN vs H p=.0540, HN vs H p=.9046; ZT14 p=.0427, LN vs HN p=.0624, LN vs H p=.076, HN vs H p=.993) and F) G6Pase mRNA as determined by RT-PCR normalized to cyclophilin and RPL13, which did not display circadian variation (n=6, ZT6 p=.0002, LN vs HN p=.0001, LN vs H p=.0188, HN vs H p=.0434; ZT10 p=.055, LN vs HN p=.997, LN vs H p=.084, HN vs H p=087; ZT18
Figure 2 Dietary iron affects the formation of the Rev-Erbα repressor complex and its activity. A) Western blot of Rev-Erbα IP extracts blotted for NCOR and Rev-Erbα at ZT14 and B) quantification of these blots (n=6, p<.0001, LN vs HN p=.0093, LN vs H p<.0001, HN vs H p<.0001 by Tukey-Kramer posthoc analysis). C) ChIP of NCOR to the PEPCK (n=4, p=.0251, LN vs HN p=.4238, LN vs H p=.02; HN vs H p=.2082) and D) G6Pase promoter determined by RT-PCR normalized to input at ZT14 (n=4, p=.0152, LN vs HN p=.4124, LN vs H p=.0121, HN vs H p=.1429).

Figure 3 Hepatic heme synthesis and steady-state levels are altered with dietary iron. A) Total heme levels as measured by HPLC at ZT12 (n=6, p=.019, LN vs HN p=.3744, LN vs H p=.0158, HN vs H p=.2601 by Tukey-Kramer posthoc analysis). B) Heme b levels as measured by hemochromagen pyridine assay at ZT12 (n=12, p=.0076, LN vs HN p=.3398, LN vs H p=.0059, HN vs H p=.1009). C) ALAS1 transcripts determined by RT-PCR normalized to cyclophilin and RPL13 (n=6, p=.0002, LN vs HN p=.083, LN vs H p=.0002, HN vs H p=.016).

Figure 4 Drug induced increase or decrease of heme levels ablates variations in heme, Rev-Erbα/NCOR complex formation, and gluconeogenesis observed between the various diets. A) Heme levels as measured by HPLC with ALA treatment and control (n=6-12, Control p=.019, LN vs HN p=.3744, LN vs H p=.0158, HN vs H p=.2601; ALA treated p=.0239, LN vs HN p=.8931, LN vs H p=.0322, LN vs H p=.0603 by Tukey-Kramer posthoc analysis). B) AUCg from ZT12 PTT, (n=6, Control p=.0336, LN vs HN p=.0625, LN vs H p=.0420, HN vs H p=.8723) C) PEPCK (n=9, p=.0042, LN vs HN p=.049, LN vs HN p=.052, LN vs H p=.041, LN vs H p=.9985), and D) G6pase mRNA transcripts in ALA treated and control mice normalized to RPL13 and cyclophilin (n=9, p<.0001, LN vs HN p=.0519, LN vs HN p<.0001, HN vs H p=.0004). E) Western blot of Rev-Erbα IP extracts blotted for NCOR and Rev-Erbα and F) quantification of the density of the NCOR blot normalized to the Rev-Erbα density (n=4-6, p<.0001, LN control vs HN control p<.0001, HN+ALA vs LN control p<.0001, LN control vs LN ALA p=.0139, LN control vs HN p=.001, LN control vs LN ALA p=.0003, LN control vs H control p<.0001, LN control vs H ALA p= p<.0001, LN ALA vs HN control p=.15, LN ALA vs LN HNA p=.0189, LN ALA vs H control p<.0001, LN ALA vs H ALA p=.0007, HN control vs HN control p=.056, HN control vs H control p=.0003, HN control vs H ALA p=.003, HN ALA vs H control p=.36, HN ALA vs H ALA p=.48, H control vs H ALA p=.86). G) Heme levels as measured by HPLC with INH treated and control mice (n=3, p=.035, LN vs HN p=.5486, LN vs H p=.029, HN vs H p=.1081). H) AUCg for ZT12 PTT (n=5-8, p=.0063, LN vs HN p=.0164, LN vs H p=.0072, HN vs H p=.7276). I) PEPCK (n=5-8, p=.0041, LN vs HN p=.0054, LN vs H p=.03, HN vs H p=.0626) and J) G6Pase transcript levels as measured by RT PCR normalized to...

Figure 5 Treatment of HepG2 cells with FAC, ALA, or INH recapitulates mouse results. A) PEPCK (n=12-24, p=.000467) and B) G6Pase transcript levels as measured by RT PCR for control and FAC cells with no drug treatment, ALA, or INH treated (n=12-24, p=.0026). C) Western blot of Rev-Erβα IP cell extract blotted for NCOR and Rev-Erβα and D) quantification of the density of the NCOR band as normalized to Rev-Erβα (n=3, p=.049).

Figure 6 Increased Dietary iron increases PGC-1α. A) PGC-1α mRNA as measured by RT PCR (n=6, ZT6 p=.036, LN vs HN p=.1485, LN vs H p=.0272, HN vs H p=.6346, ZT10 p=.0495, LN vs HN p=.5224, LN vs H p=.0472, HN vs H p=.5266). B) Western blot of liver extracts blotted for PGC-1α at ZT10 and C) Density of PGC-1α band normalized to β actin (n=6, p=.0092, LN vs HN p=.5029, LN vs H p=.0079, HN vs H p=.0734). D) Verification of PGC-1α KD by RT PCR (n=6, p=.0167) and E) PEPCK,(n=6, p=.048) F) G6pase (n=6, p=.037), and G) ALAS1 transcript levels in PGC-1α KD normalized to Rpl13 (n=6, p=.0072).

Figure 7 Dietary iron alters hepatocyte oxidant signaling and antioxidant treatment ablates differences in PGC-1α and gluconeogenic transcripts A) GC MS/MS measurement of GSH (n=6, ZT6 p=.0395, LN vs HN p=.0421, LN vs H p=.0315, HN vs H p=.27; ZT10 p=.0021, LN vs HN p=.891, LN vs H p=.004, HN vs H p=.0031; ZT14 p=.0002, LN vs HN p=.827, LN vs H p=.0002, HN vs H p=.0003; ZT18 p=.0019, LN vs HN p=.047, LN vs H p=.0023, HN vs H p=.0073; ZT22 p=.0052, LN vs HN p=.8194, LN vs H p=.0031, HN vs H p=.0048) and B) NADPH/NADP+ in freeze clamped liver of mice on iron diets at various harvest times (n=6, ZT6 p=.0371, LN vs HN p=.281; LN vs H p=.0249; HN vs H p=.0582; ZT 14 p=.023, LN vs HN p=.0385, LN vs H p=.0422, HN vs H p=.9955; ZT22 p=.0047, LN vs HN p=.0523, LN vs H p=.0494, HN vs H p=.9993). C) Fasting blood glucose measurements in control and NAC treated mice (n=5-10, p<.0001, LN vs H p=.048, HN vs H p=.02, NAC vs HN NAC p=.0258,LN NAC vs H NAC p=.0006, ). D) Transcript levels of PGC-1α (n=3-6, p=.0021, LN vs HN p=.038, LN vs H p=.0017, HN vs H p=.1752), E) PEPCK (n=3-6, p=.0043, LN vs HN p=.0031, LN vs HN p=.0025, HN vs H p=.9921), F) G6Pase (n=3-6, p=.0012, LN vs HN p=.0015, LN vs H p=.0008, HN vs H p=.061), and G) ALAS1 in NAC treated and control
mice (n=3-6, p=.0071, LN vs HN p=.1971, LN vs H p=.0076, HN vs H p=.1027). H) GC MS/MS measurement of GSH (n=3-6, No treatment p=.034, LN vs HN p=.887, LN vs H p=.0316, HN vs H p=.086; NAC p=.147, LN vs HN p=.9942, LN vs H p=.194, HN vs H p=.113) and I) NADPH/NADP+ in NAC treated or control mice at ZT12 (n=3-6, p=.029, LN vs HN p=.9790, LN vs H p=.0318, HN vs H p=.0433).