Hypoxia increases Sirtuin 1 expression in a Hypoxia Inducible Factor-dependent manner

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Hypoxia Inducible Factors (HIFs) are stress-responsive transcriptional regulators of cellular and physiological processes involved in oxygen metabolism. Although much is understood about the molecular machinery that confers HIF responsiveness to oxygen, far less is known about HIF isoform specific mechanisms of regulation, despite the fact that HIF-1 and HIF-2 exhibit distinct biological roles. We recently determined that the stress-responsive genetic regulator Sirtuin 1 (Sirt1) selectively augments HIF-2 signaling during hypoxia. However, the mechanism by which Sirt1 maintains activity during hypoxia is unknown. In this report, we demonstrate that Sirt1 gene expression increases in a HIF-dependent manner during hypoxia in Hep3B and in HT1080 cells. Decreased HIF signaling results in functional consequences for Sirt1 activity; decreased HIF-1 signaling results in the appearance of acetylated HIF-2 without pharmacological inhibition of Sirt1. We also found that Sirt1 augments HIF-2, but not HIF-1, mediated transcriptional activation of the isolated Sirt1 promoter. These data in summary reveal a direct functional link between HIF signaling and Sirt1 expression as well as activity during hypoxia.

INTRODUCTION

The ability to sense and respond to changes in oxygen content, conserved in almost all eukaryotic organisms, is conferred at the cellular level and is dictated by changes in gene expression including by de novo transcriptional events (1). Members of the Hypoxia Inducible Factor (HIF) family of transcription factors are key regulators of genes whose expression is altered during hypoxia. HIFs, obligate heterodimeric protein complexes, are composed of an oxygen labile α-subunit and a shared, oxygen-stable β-subunit also referred to as ARNT (2). Whereas invertebrates contain a single HIF α member, mammals contain three HIF-α genes: HIF-1α, HIF-2α also called Endothelial PAS domain protein 1 (EPAS1), and HIF-3α (3-5). HIF-α proteins have similar domain structures with conserved sequence identity in some regions, particularly for HIF-1α and HIF-2α. The amino termini of HIF-α and HIF-β proteins contain the highly conserved basic helix-loop-helix (bHLH) and Per/ARNT/Sim (PAS) domains involved in DNA binding and protein-protein interactions, respectively. The PAS domain may also contribute to HIF target gene specificity and may serve as a target for small molecules that disrupt specific HIF complexes (6,7).
The levels of HIF-α subunits increase during hypoxia due to impaired modifications of two proline residues (8,9) situated within the oxygen-dependent degradation domain (ODD) (10), part of a larger domain known as the amino terminal activation domain (NTAD) located in the mid-portion of HIF-α proteins. These two proline residues are otherwise selectively hydroxylated under normoxic conditions by oxygen-dependent prolyl hydroxylases (8,9,11) and subsequently target the HIF-α proteins for proteasomal degradation by the von Hippel-Lindau (VHL) ubiquitin-protein ligase complex (12-16).

A second oxygen-dependent hydroxylation by asparaginyl hydroxylases (17,18) targets an asparagine residue within the C-terminal transactivation domain (CTAD) of HIF-α under normoxic conditions, thereby blocking recruitment of the coactivators p300 and CBP to the carboxy terminus and decreasing HIF transactivation capacity (18). Mutation of the modified proline and asparagine residues results in oxygen-insensitive, “constitutively active” HIF-α proteins.

Despite the overall sequence conservation in some regions, each HIF-α protein has distinct physiological roles (19,20) that are in part conferred by target gene selectivity. The divergent portion of HIF-α that we refer to as the unique region (UR), located in the carboxy terminus between the NTAD/ODD and CTAD (21), likely participates in HIF isoform selective signaling (22). Indeed, several factors have been identified that bind in a HIF-α protein selective manner or that mediate their action through the UR of either HIF-1α (23) or HIF-2α (24-26).

Sirt1, a deacetylase initially identified in aging studies of lower eukaryotes (27) and implicated in diverse physiological processes in mammals, augments HIF-2 signaling during hypoxia in cell culture as well as mouse models (21). Augmentation of HIF-2 signaling by Sirt1 requires an intact deacetylase activity of Sirt1 and is conferred by deacetylating specific lysine residues located in the HIF-2α UR that are acetylated. Absolute levels of HIF-2α acetylation increase during hypoxia, although acetylated HIF-2α is only evident if Sirt1 activity is inhibited.

Despite the increase in HIF-2α protein levels during hypoxia, Sirt1 action is so efficient that acetylated HIF-2α is undetectable unless Sirt1 deacetylase activity is inhibited. How Sirt1 action is maintained, or increased, during hypoxia is unclear. In this study, we asked if Sirt1 expression was itself altered during hypoxia. We noted that Sirt1 gene expression in cells as well as in mice increased during hypoxia and determined that HIF signaling was primarily responsible for the increased Sirt1 expression during hypoxia.

**EXPERIMENTAL PROCEDURES**

### Reporter and expression plasmids.

The human SIRT1 promoter region, (-354/+54), was isolated by PCR amplification of human genomic DNA and inserted into the firefly luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI). We introduced mutations of HIF-responsive element 5 (HRE5) or flanking ETS-binding sites (EBS) into the (-354/+54) SIRT1 promoter using PCR-based site-directed mutagenesis (QuikChange II; Stratagene) or extension PCR mutagenesis. We previously described the oxygen-independent (PPN) HIF-1α, PPN HIF-2α, wild-type (WT, W) vsv-g:SIRT1, and deacetylase-mutant (DAC, D) vsv-g:SIRT1 expression vectors (21).

### siRNA knockdown.

The day before transfection, we trypsinized and plated 2.0 x 10^5 Hep3B cells on each well of a 6-well plate in 2 mL antibiotic-free complete DMEM medium. Using DharmaFECT1 (Cat. No. T-2001-03, Thermo Fisher Scientific, Lafayette, CO), we transfected non-targeting control (Cat. No. D-001810-10-20, Thermo Fisher Scientific, Lafayette, CO), HIF-1α (Cat. No. L-004018-
siRNA. After 24 or 48 hours, cells were harvested for mRNA or protein analysis, respectively.

**Immunoblotting and cell fractionation.**
To prepare nuclear extracts from Hep3B or HT1080 cells, we used NE-PER® nuclear and cytoplasmic extraction reagents (Cat. No. 78833, Pierce, Rockford, IL). To prepare whole cell extracts from Hep3B or HT1080 cells, we used cytoBuster protein extraction reagent (Cat. No. 71009, Novagen, Gibbstown, NJ). Equivalent amounts of whole cell extracts or nuclear extracts in SDS-PAGE sample buffer were analyzed by immunoblotting using anti-human Sirt1 (1:1,000 dilution; Cat. No. 07131, Upstate Biotechnology, Inc.), anti-human HIF-1α (1:1,000 dilution; Cat. No. 610959, BD Biosciences, Franklin Lakes, NJ), anti-human HIF-2α (1:1,000 dilution; Cat. No. NB100-132, Novus Biologicals, Littleton, CO), anti-HA (1:5,000 dilution; Cat. No. H9658, Sigma), anti-vsv-g antibodies (1:5,000 dilution; Cat. No. ab18612, Abcam, Cambridge, MA), anti-α-tubulin (1:5,000 dilution; Cat. No. T5168, Sigma), or anti-TATA-binding protein (TBP) (1:1,000 dilution; Cat. No. sc-204, Santa Cruz Biotechnology) antibodies.

**Detection of exogenous HIF-2α acetylation.**
Hep3B or HT1080 cells were transfected with expression vectors encoding amino-terminal S-peptide (SP) epitope tagged and carboxy-terminal hemagglutinin A (HA) epitope tagged P1P2N HIF-1α (SP:P1P2N HIF-1α:HA) or P1P2N HIF-2α (SP:P1P2N HIF-2α:HA) as previously described (21). Twenty-four hours after transfection, cells were exposed to hypoxia up to 24 hr. Eight hours before harvesting, cells were treated with 5 µM sirtinol plus 10 mM nicotinamide (NAM). After harvesting, SP:P1P2N HIF-2α:HA and SP:P1P2N HIF-2α:HA were purified for immunoblot analyses of acetylated lysine residues or the HA epitope as previously described (21).

**Cell culture and transfections.**
We used the indicated amounts of reporter plasmids (30 ng/well) and expression plasmids (100 ng/well) in transient transfection analyses. We maintained human renal clear cell carcinoma 786-0 (Cat. No. CRL-1932, ATCC), human fibrosarcoma HT1080 (Cat. No. CCL-121, ATCC), and human hepatocellular carcinoma Hep3B (Cat. No. HB-8064, ATCC) cells in complete media [DMEM, 4.5 g/l glucose, 4 mM glutamate (Cat. No. SH30022, HyClone, Logan, UT), 10% fetal bovine serum (FBS; Cat. No. S10650H, Atlanta Biologicals, Lawrenceville, GA) with penicillin (100U/mL)/streptomycin (100 µg/mL) (Cat. No. 15140-148, Gibco BRL, Carlsbad, CA)] in a 5% CO₂, 95% N₂ incubator. We transfected Hep3B or HT1080 cells in 48-well plates (Cat. No. 3548, Corning Inc., Lowell, MA) at 50-60% confluence using Lipofectamine 2000 (Cat. No.11668-019, Invitrogen) and made up the balance with pIREs. At 24 hr post-transfection, we harvested cells for luciferase assays. For hypoxia treatments, we transferred cells to a humidified environmental chamber (Coy Laboratory Products, Inc., Grass Lake, MI), replaced culture media with deoxygenated media, and maintained the cells under hypoxic (1% O₂, 5% CO₂, 94% N₂) conditions for the specified periods. We prepared cell extracts within the chamber.

**Chromatin immunoprecipitation assays in cells.**
We seeded Hep3B cells at 2 x 10⁶ (150 mm plates) 48 hr prior to use, next exposed the cells to normoxia or hypoxia, and then harvested for whole cell protein or RNA. EPO induction after hypoxia exposure was confirmed by real-time RTPCR. Chromatin immunoprecipitation (ChIP) assays were
carried out using the ChIP-IT™ Express Magnetic assay kit (Cat. No. 53009, Active Motif, CA). The antisera used were normal mouse IgG (1-2 µg/ml; Cat. No. 2027, Santa Cruz Biotechnology), normal rabbit IgG (1-2 mg/ml; Cat. No. NI01, EMD Chemicals, Inc., Gibbstown, NJ), anti-human EPAS1 antiserum (2 mg/ml; NB 100-132, Novus Biologicals), and mouse anti-human HIF-1α (2 mg/ml Cat. No. 610958, BD Biosciences, San Diego, CA). After ChIP, the precipitated genomic DNA was analyzed by quantitative PCR using an Applied Biosystems ABI Prism 7000 thermocycler (Applied Biosystems; Foster City, CA), Power SYBR Green Master Mix (Cat. No. 4367659, Applied Biosystems), and the following primers: 5’-GGCAACAGGCCCCGAGGGCTGGCTTGG GCA-3’ (mouse Sirt1 promoter forward) and 5’-TCTTCCAACTGCCTCTGCCCCTCCGC CC-3’ (mouse Sirt1 promoter reverse). The captured genomic DNA was normalized to input material and compared between the normoxic and hypoxic treatment samples with results of a representative experiment shown.

Chromatin immunoprecipitation assays in mice.

CD1 mice were exposed to 6% oxygen for 2 hr, and then euthanatized for harvest of liver samples inside a hypoxia chamber. We minced 30 mg of fresh tissue to 1-3 mm³, transferred tissue into 10 mL PBS plus protease inhibitors/gram of tissue, added formaldehyde (final concentration 1%), and rotated tubes at room temperature for 15 minutes. We stopped cross-linking with fresh glycine (final concentration of 0.125 M). After five minutes at room temperature, the tissue was pelleted at low speed centrifugation at 4°C, washed once with cold PBS plus protease inhibitors, and then repelleted. We resuspended the washed pellet in 1 mL of PBS on ice and ground the tissue using a micro-tissue grinder on ice. Cells were pelleted by micro-centrifugation at 4°C. Chromatin immunoprecipitation (ChIP) was carried out using the ChIP-IT™ Express Magnetic assay kit (Cat. No. 53009, Active Motif, CA). After ChIP, the precipitated genomic DNA was analyzed for the presence of a mouse Sirt1 amplicon by quantitative PCR using an Applied Biosystems ABI Prism 7000 thermocycler (Applied Biosystems; Foster City, CA), Power SYBR Green Master Mix (Cat. No. 4367659, Applied Biosystems), and the following primers: 5’-AGCAAGGACGAGAAAAAGGAGCAAAA GAGGAG-3’ (forward) and 5’TCTTCCAACTGCCTCTGCCCCTCCGC CC-3’ (reverse). The captured genomic DNA was normalized to input material and compared between the normoxic and hypoxic treatment samples.

Real time RT-PCR analyses.

For cells, total RNA was extracted using GenElute™ mammalian Total RNA kit (Cat. No. RTN70-1KT, Sigma). For mouse liver, total RNA was isolated from ~1/8 of the mouse liver (right lobule) and extracted using the FastRNA Pro Green Kit (Cat. No. 6045, MP Biomedicals, Solon, OH). 1 µg of total RNA was reverse transcribed with oligo-dT primers using a M-MLV reverse transcriptase Kit (Cat. No.28025-013, Invitrogen). Real time quantitative PCR was performed on an Applied Biosystems ABI Prism 7000 thermocycler using Power SYBR Green Master Mix following the manufacturer’s protocol and one-tenth of total cDNA with the following pairs of human primers: CYCLOPHILIN B (forward) 5’-ATGTGGTTTTTCGGAAATGCTA-3’, CYCLOPHILIN B (reverse) 5’-GGCTTGTCCCAGCTTGCTT-3’, SIRT1 (forward) 5’-GCAGGTTGGAGGAATCCAA-3’, SIRT1 (reverse) 5’-GGCAAGATGCTGTTGCAAA-3’, PGK1 (forward) 5’-TTAAAGGGAAGCGGCTGTTA-3’, PGK1 (reverse) 5’-
TCCATTGTCCAAGCAGAATTTGA-3’,
EPO (forward) 5’-
GAGGCCGAGAATATCACGACGGG-3’,
EPO (reverse) 5’-
TGCCCGACCTCCATCTTCTCCAG-3’; or
mouse primers: cyclophilin B (forward) 5’-
ATGTGGTTTTCGGCAAAGTTCTA-3’,
cyclophilin B (reverse) 5’-
GGCTTGTCCCGGCTGTCT-3’, Sirt1
(forward) 5’-
GCAGGTTGCAGGAATCCAA-3’,
Sirt1 (reverse) 5’- GGCAAGATGCTGTTGCAAA-
3’. We expressed the results of triplicate
experiments as 2 (-(EPO number of cycles - cyclophilin
number of cycles)). Levels for genes of interest were
normalized to cyclophilin B mRNA.

Mouse hypoxia experiments.
All mice were housed under standard 12 hr:12
hr light:dark conditions and fed ad lib with
standard chow. We established C57BL/6J
HIF-1α heterozygous (28) x 129S6/SvEvTac
HIF-2α (29,30) heterozygous mating pairs for
generation of F1 hybrid
(129S6/SvEvTac:C57BL/6J) wild-type, HIF-
1α heterozygous, HIF-2α heterozygous, and
HIF-1α/HIF-2α compound heterozygous mice.
For hypoxic experiments, we placed age- and
gender-matched wild-type CD1 or wild-type,
HIF-1α, HIF-2α, and HIF-1α/HIF-2α compound heterozygous F1 hybrid mice in a
hypoxia chamber with flow by air supply and
subjected to normoxic (21% oxygen) or
continuous hypoxic (6% oxygen) treatment for
2 hours. Mice were then euthanized, tissues
collected, and snap-frozen in liquid nitrogen
for subsequent extraction of total RNA. Real-
time RTPCR determinations were made for
genes of interest as described.

Mouse adenoviral experiments.
We transduced adult CD1 wild-type female
mice (Charles River Laboratories; Wilmington,
MA) with PPN HIF-1α:HA, PPN HIF-2α:HA,
or control (GFP) adenovirus as described (21).
Hepatic Sirt1 and Cyclophilin mRNA levels
were determined by real-time RTPCR
analyses.

Statistical analyses.
Statistical analyses were performed using
Microsoft Excel (Microsoft Corporation,
Redmond, WA) and StatPlus:mac LE
(AnalystSoft, Inc.) by Student’s t-test or by
ANOVA as indicated. One-tailed analyses
were performed as we anticipated reductions
or increases in HIF signaling would blunt or
augment Sirt1 expression, respectively. P
values less than 0.10 were deemed statistically
significant.

RESULTS

Sirt1 expression increases during hypoxia.
The activity of Sirt1 can be controlled by
transcriptional as well as by post-translational
mechanisms. Recently, we determined that
Sirt1 selectively augmented HIF-2α signaling
during hypoxia. We surmised that the
participation of Sirt1 in HIF-2 signaling
during hypoxia reflects a physiologically
relevant interaction of two crucial stress-
responsive signaling pathways. However, the
mechanism by which Sirt1 activity is
maintained or even increased during hypoxia
was unknown. We hypothesized that Sirt1
regulation during hypoxia could be conferred
by changes in Sirt1 gene expression.
We first performed a time-course analysis
of acetylation during hypoxia in Hep3B cells,
a model hypoxia-responsive cell line, as well
as in HT1080 cells (Fig. 1A), which have
recently been used in Sirt1/HIF studies (31).
Ectopic HIF-1α and HIF-2α exhibited marked
differences in the ability to undergo
acetylation during hypoxia in both cell lines.
HIF-2α, but not HIF-1α, was efficiently
acetylated within 2 hr, peaked by 4 to 8 hr,
and returned to baseline values by 24 hr
following hypoxia exposure.

We next examined SIRT1 protein levels
during hypoxia in Hep3B and in HT1080 cells
(Fig. 1B). Immunoblotting of SIRT1 revealed
a time-dependent increase in protein levels for both cell lines with the peak of expression at 4 hr following hypoxia exposure. To determine whether the observed changes in SIRT1 protein levels during hypoxia were due to changes in SIRT1 gene expression, we measured steady-state SIRT1 mRNA levels by real-time RTPCR (Fig. 1C) in parallel samples as assessed in Fig. 1B. SIRT1 mRNA levels increased in a time-dependent manner during hypoxia for both Hep3B as well as HT1080 cells and peaked at the same time-point (4 hr) as observed for the peak of SIRT1 protein expression.

HIF-α members activate and are recruited to the Sirt1 proximal promoter.

Because SIRT1 gene expression increased during hypoxia, we hypothesized that HIF members might directly participate in the increase in SIRT1 gene expression during hypoxia. The human proximal SIRT1 promoter region contains several candidate HIF-responsive elements (HREs) conserved between the mouse and human promoters that could potentially serve as functional transcriptional regulatory elements (Fig. 2A). One of these candidate HREs, HRE5, also is flanked by candidate ETS-binding sites (EBS), which have previously been shown to confer HIF-2 selective activation (32-34). To test whether the proximal SIRT1 promoter would respond to HIF activation, we isolated the human SIRT1 proximal promoter and fused it to firefly luciferase coding sequences for use in transient transfection assays with Hep3B and HT1080 cells.

To evaluate the effect of HIF signaling on the isolated SIRT1 promoter, we over-expressed oxygen-independent (PPN) mutant forms of HIF-1α or HIF-2α that are not modified by the oxygen-dependent prolyl or asparaginyl hydroxylases (Fig. 2B). These constructs allow for HIF signaling during normoxia, thereby avoiding signaling induced by other hypoxia-activated regulators. Both PPN HIF-1α and PPN HIF-2α increased activity of the isolated SIRT1 promoter reporter in both Hep3B as well as in HT1080 cells (Fig. 2B). Reporter activity was more pronounced with PPN HIF-2α compared to PPN HIF-1α over-expression, despite equivalent or even greater levels of PPN HIF-1α (see Figure 1A, 0 hr hypoxia time-point). Moreover, SIRT1 co-expression augmented HIF-2α, but not HIF-1α, stimulation of the SIRT1 proximal promoter reporter.

HIF signaling is primarily mediated through binding of HIF heterodimers to HIF-responsive elements (HREs) in regulatory regions of HIF target genes, although additional cis-elements may influence responsiveness of a given HRE to either HIF-1 or HIF-2. We hypothesized that HRE5 in the proximal SIRT1 promoter region might mediate HIF signaling, given its location in a typical enhancer position and the presence of flanking EBS that are important for HIF-2 signaling. To specifically evaluate the contribution of HRE5 to HIF signaling, we generated a point mutation of HRE5 in the (-354/+54) SIRT1 promoter and observed a profound effect on SIRT1 promoter activity such that it was rendered unresponsive to either PPN HIF-1α or PPN HIF-2α (Fig. 2B). These data indicate that HRE5 is likely a functional and crucial HRE conferring HIF-mediated induction of SIRT1 gene expression during hypoxia.

To determine whether the EBS that flank HRE5 contribute to HIF isoform activation of the SIRT1 promoter, we constructed and tested a SIRT1 promoter point mutant that lacked functional flanking EBS. As seen (Fig. 2B), ablation of the flanking EBS blunts fold-induction by PPN HIF-2α, but not by PPN HIF-1α. However, the EBS mutations do not prevent augmentation of PPN HIF-2α signaling by SIRT1. Similar to our results with other HIF-responsive regulatory regions in HEK293 and Hep3B cells (21), we did not observe an effect of SIRT1 on induction by...
PPN HIF-1α of isolated SIRT1 promoter activity in either Hep3B or HT1080 cells.

We next asked whether HIF-1α or HIF-2α are recruited to the endogenous SIRT1 promoter during hypoxia. We performed chromatin immunoprecipitation (ChIP) assays using extracts from Hep3B cells exposed to various periods of hypoxia with a PCR amplicon that centered on HRE5 of the SIRT1 promoter region (Fig. 2C). As shown, endogenous HIF-1α and HIF-2α are recruited to the endogenous SIRT1 proximal promoter region during hypoxia (Fig. 2C).

HIF-1α and HIF-2α contribute to Sirt1 induction during hypoxia in cells.

Because exogenous PPN HIF-1α as well as PPN HIF-2α increased activity of the isolated SIRT1 promoter in transient transfection assays, we asked if pathological states involving up-regulation of HIF alpha members would result in increases in SIRT1 mRNA and SIRT1 protein levels. We began with examination of Sirt1 expression in 786-0 cells, derived from von Hippel Lindau (VHL) tumors in which the inactivation of the VHL protein results in constitutive HIF alpha expression, after transfection with control or VHL-encoding transposons. In the absence of functional VHL protein, HIF-1α or HIF-2α proteins are stabilized under normoxic conditions in 786-0 cells (Fig. 3B). Introduction of a functional VHL protein resulted in the expected decrease in HIF-1α and HIF-2α proteins and was accompanied by a reduction of SIRT1 mRNA (Fig. 3A) and SIRT1 protein (Fig. 3B) levels.

Because VHL loss may affect other signaling pathways besides HIF, we asked if HIF signaling alone, as conferred by exogenous PPN HIF-1α or PPN HIF-2α overexpression, could affect Sirt1 levels different in Hep3B cells. Exogenous PPN HIF-1α as well as PPN HIF-2α significantly increased expression of endogenous SIRT1 mRNA (Fig. 3C) and SIRT1 protein (Fig. 3D) levels in Hep3B cells. Expression of exogenous PPN HIF-2α resulted in a similar increase in Sirt1 gene expression as the increase observed with exogenous PPN HIF-1α, even though ectopic expression of exogenous PPN HIF-2α was less than exogenous PPN HIF-1α.

We next asked whether knockdown of endogenous HIF-α subunits affects endogenous Sirt1 induction during hypoxia. Following introduction of siRNA recognizing either HIF-1α or HIF-2α, Hep3B cells were exposed to normoxia or hypoxia and total RNA prepared. Knockdown of HIF-1α trended towards lower induction and knockdown of HIF-2α significantly blunted induction of SIRT1 mRNA during hypoxia, but had no effect in cells maintained under normoxic conditions (Fig. 3E). These reductions in SIRT1 mRNA levels were not a general inhibitory effect of HIF knockdown; the HIF-1 target gene PGK1 was only reduced after HIF-1α knockdown whereas the HIF-2 preferential target gene EPO was predominantly affected after HIF-2α (Supplemental Information, Fig. S1A-B). The reduction in SIRT1 mRNA levels resulting from HIF-1α or HIF-2α knockdown was accompanied by a blunting of the increase in SIRT1 protein levels during hypoxia, but did not affect SIRT1 protein levels during normoxia (Fig. 3F).

HIF members regulate Sirt1 gene expression in mice.

We have previously shown that ectopic expression of PPN HIF-1α or PPN HIF-2α in the livers of mice results in activation of HIF-1 or HIF-2 target genes. We now asked if Sirt1 levels differed in mice expressing ectopic PPN HIF-1α or PPN HIF-2α. Similar to results obtained in Hep3B cells (Fig. 3C-D), ectopic PPN HIF-1α or PPN HIF-2α expression in mouse livers significantly increased endogenous liver Sirt1 mRNA (Fig. 4A) and Sirt1 protein (Fig. 4B) levels.
Furthermore, and as seen in Hep3B cells, ectopic PPN HIF-1α resulted in a similar increase in Sirt1 protein levels as observed with ectopic PPN HIF-2α, despite the lower levels of the latter compared to the former ectopic HIF-α protein.

Because Sirt1 gene expression increases in cells during hypoxia, we next asked whether endogenous Sirt1 mRNA and Sirt1 protein levels were altered in livers of mice exposed to hypoxia. Hepatic Sirt1 mRNA (Fig. 4C) and protein (Fig. 4D) levels increased during hypoxia exposure relative to mice maintained under normoxic conditions. This was accompanied by recruitment of endogenous HIF-1α and HIF-2α to the Sirt1 promoter region in liver after hypoxia exposure as revealed by in vivo chromatin immunoprecipitation assays (Fig. 4E). The importance of HIF members for induction of Sirt1 gene expression was demonstrated by the blunted induction of Sirt1 mRNA in mice that were haploinsufficient for HIF-1α, HIF-2α, or both HIF-1α and HIF-2α (Fig. 4F).

Impairment of HIF signaling has functional consequences on Sirt1 activity.

Sirt1 gene expression increases during hypoxia, but increase is blunted by reduction in either HIF-1α or HIF-2α protein levels. We have previously shown that Sirt1 deacetylates HIF-2α. We next asked if a reduction in Sirt1 levels conferred by HIF-1α knockdown would affect the acetylation status of HIF-2α.

We first confirmed that knockdown of HIF-1α resulted in decreased SIRT1 levels. Reduction of SIRT1 protein was evident when HIF-1α was knocked down (Fig. 5A, lane 2) and was unaffected by inhibition of SIRT1 activity mediated by the pharmacological inhibitors sirtinol and nicotinamide (NAM) (Fig. 5A, lane 4). Ectopic expression of wild-type (Fig. 5A, lane 6) or deacetylase inactive (Fig. 5A, lane 8) SIRT1 did not affect HIF-1α knockdown.

We examined whether a reduction in endogenous Sirt1 protein levels, as conferred with HIF-1α knockdown, affected acetylation of endogenous HIF-2α during hypoxia. Acetylated HIF-2α is normally undetectable without Sirt1 inhibition (Fig. 5B, lane 1). However, after knockdown of HIF-1α, acetylated HIF-2α was readily detectable in the absence of the pharmacological inhibitors (Fig. 5B, lane 2). This was not due to an overall increase in acetylated HIF-2α levels as addition of Sirt1 pharmacological inhibitors resulted in similar levels of acetylated HIF-2α in the absence (Fig. 5B, lane 3) or presence (Fig. 5B, lane 4) of HIF-1α knockdown. Moreover, acetylated HIF-2α, which was detectable after HIF-1α knockdown, was deacetylated by ectopic wild-type Sirt1 (Fig. 5B, lane 6), but not by deacetylase mutant (Fig. 5B, lane 8) Sirt1.

DISCUSSION

Why is it important to understand how Sirt1 action is maintained during hypoxia? Studies of Sirt1 activation to date have primarily focused on post-translational mechanisms of regulation. These mechanisms include changes in pyridine nucleotide levels or ratios that affect Sirt1 enzymatic activity, Sirt1 phosphorylation or other kinase-induced effects (35-40), or Sirt1 subcellular localization (41-44). Changes in pyridine nucleotide levels or ratios are perhaps the best-studied mechanism for regulating Sirt1 function (45). However, this mechanism is unlikely to be responsible for activation of Sirt1 during hypoxia.

During ischemia, the change in redox state, which mirrors the pyridine nucleotide ratio, does not favor Sirt1 activation. Similarly, the alterations in pyridine nucleotide levels during hypoxia are opposite that associated with increased Sirt1 enzymatic activity. Most hypoxia/pyridine nucleotide studies examined total NAD+ or NADH levels rather than free...
NAD+ or NADH levels or ratios, which are the relevant molecular species for NAD+/NADH regulated enzymes such as Sirt1. Nevertheless, it is unlikely that a major change in direction of the cellular redox state during hypoxia would occur if free NAD+/free NADH ratios were calculated. Therefore, these studies suggest an alternative mechanism for activation of Sirt1 during hypoxia besides changes in redox state and pyridine nucleotide levels/ratios.

Transcription factors that participate in hypoxia-dependent regulation, and that may be regulated by Sirt1, are attractive candidates for controlling Sirt1 activity during hypoxia. Sirt1 and HIF are critical regulators of physiological processes in mammals, including several affected by hypoxia or ischemia (46) such as metabolic adaptation (47-49), angiogenesis (50-55), and oxidative stress defense mechanisms (56). A logical expectation is that feedback mechanisms may exist for transcription factors whose target genes encode genetic regulators, which in turn act upon the transcription factor itself. This occurs with FoxO, a transcription factor whose activity is also augmented by Sirt1 deacetylation (57) and which acts as a positive regulator of Sirt1 gene expression (58).

Based upon our data obtained from cell culture and animal models, we suggest that Sirt1 induction during acute hypoxia is mediated through binding of HIF complexes to, and subsequent activation of, the proximal Sirt1 promoter (Fig. 5C). An increase in either HIF-1 or HIF-2 signaling is sufficient to increase Sirt1 gene expression whereas a decrease in either HIF-1 or HIF-2 signaling blunts the induction of Sirt1 gene expression during acute hypoxia. Finally, although Sirt1 acts selectively with HIF-2α to augment HIF-2 signaling, Sirt1 does not augment HIF-1 induction of isolated HIF-responsive regulatory regions from the Epo enhancer, VEGF promoter, Sod2 promoter and, as now reported, the Sirt1 proximal promoter in Hep3B cells (21) raising the possibility of positive feedback regulation.

As with other HIF regulated regulatory regions, we only observed a selective and stimulatory action of Sirt1 on HIF-2, and not HIF-1, transactivation of the Sirt1 promoter. The link between the Sirt1 and HIF-2 pathways involves Sirt1-dependent deacetylation of acetylated HIF-2α; we have previously found significant acetylation, as well as stable interactions with Sirt1, of HIF-2α, and not HIF-1α, using either exogenous or endogenous HIF-α proteins in HEK293 and Hep3B cells (21). Determining how Sirt1 deacetylation and HIF-2α acetylation cooperate to increase HIF-2 signaling will require additional studies.

Whether Sirt1 plays a role in HIF-1 signaling during acute hypoxia is less clear. We have only found a functional and positive role for Sirt1 in HIF-2, and not HIF-1, dependent induction of endogenous targets genes during acute hypoxia in Hep3B cells and in mice (21). In the current study, we extended our evaluation of HIF acetylation in a more prolonged hypoxia time-course in both Hep3B cells as well as in HT1080 cells, the cell line used in the study that reported HIF-1α acetylation (31). In contrast to the results of Lim et al., we found no significant acetylation of ectopic HIF-1α in either Hep3B or HT1080 cells during hypoxia.

The reasons for the discrepancy between our results and that of Lim et al. is unclear, although we note that Lim et al. evaluated HIF-1α acetylation sites induced by ectopic PCAF over-expression rather than by hypoxia. Because it is likely more robust, acetylation induced by exogenous PCAF may allow for easily detectable amounts of acetylated HIF-1α. However, this raises the question as to whether low-level acetylation of HIF-1α during hypoxia, if present, is physiological relevant. Irrespective of whether or not low-level acetylation of HIF-1α occurs during hypoxia, we have not observed a functional
role for Sirt1 in HIF-1 signaling in cells or in mice exposed to acute hypoxia (21). Additional experiments will be needed to clarify if Sirt1 plays a role in HIF-1 signaling during chronic hypoxia.

Our data suggest a functional role for both HIF-1 and HIF-2 in the control of Sirt1 gene expression during acute hypoxia that is mediated by transcriptional activation of the Sirt1 locus. Moreover, impeding HIF-1 signaling has functional consequences upon HIF-2 acetylation, which is evident after HIF-1α knockdown in the absence of Sirt1 inhibitors. The reduction in Sirt1 protein levels, the increase in HIF-2α acetylation due to reduced Sirt1 levels, and the lack of compensatory changes in Sirt1 gene expression by HIF-2α after HIF-1α knockdown indicate that impeding HIF-1 signaling will likely directly affect Sirt1 as well as HIF-2 signaling, thereby intricately linking Sirt1 and HIF signaling. Indeed, the blunting of EPO gene expression in Hep3B cells after HIF-1α knockdown, albeit a more muted effect compared to that resulting from HIF-2α knockdown, might reflect loss of Sirt1 augmentation of HIF-2 signaling in addition, or as opposed, to a reduction in HIF-1 mediated induction of EPO directly.

The sensitivity of Sirt1 gene expression to HIF-α gene dosage indicates an exquisite reliance upon HIF signaling, one that results in an increase in Sirt1 activity during initial hypoxia exposure. Similar to other HIF-2 responsive regulatory regions (21), HIF-2 activation of the isolated Sirt1 promoter is augmented by Sirt1. HIF-2 activation of the Sirt1 promoter is likely influenced by other trans-acting factors, besides Sirt1, as mutations in the candidate ETS-binding sites (EBS) that flank HRE5 selectively affect HIF-2, and not HIF-1, mediated induction of the isolated Sirt1 promoter. The spatial relationship of the HRE and flanking EBS are consistent with that found in other HIF-2 selective HIF target genes (32-34). Definitive proof of a role for Ets family members in Sirt1 regulation will require further studies.

With continued hypoxic exposure beyond 8 to 12 hours in cell culture, Sirt1 mRNA and protein levels begin to decline. The biphasic response of Sirt1 to hypoxia suggests a favorable role for Sirt1 in the acute phase of hypoxia and a neutral, or even deleterious, role for Sirt1 in the latter phases of hypoxia. Although the complexities of Sirt1 regulation during hypoxia have not been completely elucidated, redox-dependent mechanisms, which repress Sirt1 gene expression in a HIF-independent manner, may be relevant in the chronic hypoxia state (59).

Although we cannot exclude post-translational mechanisms as contributions to Sirt1 activation during hypoxia, our results suggest that Sirt1 activity is maintained, and even increased, during acute hypoxia through mass action effects mediated by HIF-dependent increases in Sirt1 gene expression. HIF and Sirt1 activity can be increased independent of oxygen-availability, including by oxidative stress, although these events may also affect HIF-α subunit accumulation and activation. Given the role of HIF-2 in induction of Sirt1 gene expression and the augmentation of HIF-2 signaling by Sirt1, these data support a feedback mechanism of Sirt1 activation with HIF-2 signaling during acute hypoxia and likely other environmental stress states.
ACKNOWLEDGEMENTS

We thank A. Das and E. Ballard for technical assistance, M. Alexander for suggestions for in vivo chromatin immunoprecipitation assays, Ko Uyeda for insightful comments regarding measurements of cellular redox state and their current limitations, Rick Bruick for the VHL expression plasmid, and Randall Johnson for HIF-1α founder mice. These studies were supported by the American Heart Association and the Department of Veterans Affairs.
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FOOTNOTES

The abbreviations used are: ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic-Helix-Loop-Helix; CTAD, carboxy (C)-terminal activation domain; HIF, Hypoxia Inducible Factor; HRE, HIF-response element; NTAD, amino (N)-terminal activation domain; ODD, oxygen-dependent degradation; PAS, Per/ARNT/Sim; Sirt1, Sirtuin 1; UR, unique region; VHL, von Hippel-Lindau tumor suppressor protein
Fig. 1. Sirt1 expression increases during hypoxia.
A, acetylation time-course of ectopic HIF-1α and HIF-2α expressed in Hep3B or HT1080 cells exposed to hypoxia. Top panel for each cell line is immunoblotting (IB) of acetyl-lysine residues in whole cell extract (WCE) after SP-affinity purification of ectopic HIF-1α and HIF-2α from cells exposed to hypoxia (1% oxygen) for the indicated period of time. Bottom panel is same membrane reprobed for HA epitope tag. B, immunoblot (IB) analyses of human SIRT1 or TATA-binding protein (TBP) in nuclear extract (NE) from Hep3B or HT1080 cells exposed to hypoxia (1% oxygen) for the indicated period of time. C, SIRT1 mRNA levels as measured by real-time RTPCR analyses of total RNA obtained from Hep3B or HT1080 cells exposed to hypoxia (1% oxygen) for the indicated period of time. SIRT1 mRNA levels were normalized to cyclophilin B (cyclophilin) mRNA levels for each sample. The data represent the average of three independent samples obtained at each time-point with each sample a mean of triplicate measurements. Error bars indicate standard error of the means. P=0.00002 for Hep3B samples and P=0.006 for HT1080 samples with comparisons made by one-way ANOVA.

Fig. 2. HIF activates the Sirt1 promoter.
A, schematic representation of human Sirt1 proximal promoter region. The HIF-responsive elements (HRE) conserved between mouse and human in sequence and relative position are shown by black boxes whereas HREs unique to the human SIRT1 promoter is shown as a white box. The sequences for HRE5 and surrounding ETS-binding sites (EBS) are shown in the parental wild-type (WT) construct as well as for the site-directed mutants in HRE5 (mutHRE) or in the flanking EBS (mutEBS). The nucleotide substitutions in the core HRE (ACGTG) or in the core EBS (GGA) sequences are underlined. B, transient transfection assays of the isolated human SIRT1 proximal promoter in Hep3B or HT1080 cells. Transfections were performed with the indicated isolated SIRT1 proximal promoter reporter and expression plasmids encoding no protein (–; basal values), oxygen-insensitive (PPN) HIF-1α or PPN HIF-2α along with control (–), wild-type (W), or deacetylase mutant (D) SIRT1 expression plasmids. Error bars indicate standard error of the means. For Hep3B cells, P=0.048 for PPN HIF-2α versus PPN HIF-2α + wild-type Sirt1 with wild-type SIRT1 promoter, and P=0.001 for PPN HIF-2α versus PPN HIF-2α + wild-type Sirt1 with mutEBS SIRT1 promoter. For HT1080 cells, P=0.0004 for PPN HIF-2α versus PPN HIF-2α + wild-type Sirt1 with wild-type SIRT1 promoter, and P=0.0003 for PPN HIF-2α versus PPN HIF-2α + wild-type Sirt1 with mutEBS SIRT1 promoter. All comparisons were made by one-tailed t-test. C, chromatin immunoprecipitation (ChIP) assays of HIF protein binding to the SIRT1 proximal promoter region. Binding of HIF-1α and HIF-2α to the SIRT1 proximal promoter region in Hep3B cells during hypoxia as assessed by ChIP assays is shown with a PCR amplicon centered on HRE5. The cell extracts from each time-point were subjected to ChIP assays using normal, anti-human HIF-1α, or anti-human HIF-2α immunoglobulin G (IgG).

Fig. 3. HIF activates endogenous Sirt1 gene expression in cells.
A, SIRT1 mRNA levels in 786-0 cells transfected with a control or VHL-encoding expression plasmid. SIRT1 mRNA levels were analyzed and presented as in Fig. 1A. P=0.037 by one-tailed t-test. B, immunoblot analyses for SIRT1, HIF-1α, and HIF-2α in 786-0 cells treated in parallel as in A. C, SIRT1 mRNA levels in Hep3B cells after transfection with expression plasmids encoding control (–), or oxygen-insensitive (PPN) HIF-1α or HIF-2α. P=0.024 for control versus PPN HIF-1α, and P=0.034 for control versus PPN HIF-2α. All comparisons made by one-tailed
t-test. D, immunoblot analyses for SIRT1, HIF-1α, and HIF-2α in Hep3B cells treated in parallel as in C. E, SIRT1 mRNA levels in Hep3B cells after transfection with control, HIF-1α, or HIF-2α siRNA. P=0.123 for control versus HIF-1α siRNA, and P=0.059 for control versus HIF-2α siRNA. All comparisons made by one-tailed t-test. F, immunoblot analyses for SIRT1, HIF-1α, and HIF-2α in Hep3B cells treated in parallel as in E.

**Fig. 4. HIF activates endogenous Sirt1 gene expression in mice.**

A, CD1 mice transduced with control (−) adenovirus or adenovirus encoding PPN HIF-1α:HA or PPN HIF-2α:HA. Livers were isolated 7 d following transduction for determination of Sirt1 and cyclophilin B levels from total RNA. P=0.022 for control versus PPN HIF-1α, and P=0.019 for control versus PPN HIF-2α. All comparisons made by one-tailed t-test. B, immunoblot analyses of Sirt1 and α-tubulin expression in livers isolated from three independent mice for each group examined in A. C, CD1 mice exposed to room air (21% oxygen) or continuous hypoxia (6% oxygen) for 2 h followed by harvesting of livers for total RNA preparation. Sirt1 and cyclophilin B mRNA levels were determined as in A. P=0.004 for normoxia versus hypoxia by one-tailed t-test. D, immunoblot analyses of Sirt1 and α-tubulin expression in livers isolated from three independent mice for each group examined in C. E, schematic representation of the mouse Sirt1 proximal promoter region as well as the amplicon centered on HRE5 and used in chromatin immunoprecipitation (ChIP) assays of HIF binding to the Sirt1 proximal promoter region. Binding of HIF-1α and HIF-2α to the Sirt1 proximal promoter region, as assessed by ChIP assays, in mice maintained under normoxia or exposed to hypoxia is shown. No signal was evident with control immunoglobulin (IgG) under either condition. F, Mice with intact (WT) HIF levels or haploinsufficient for HIF-1α (HIF-1α+/−), HIF-2α (HIF-2α+/−), or both HIF-2α and HIF-2α (HIF-1α+/−/HIF-2α+/−) were exposed to room air (21% oxygen) or continuous hypoxia (6% oxygen) for 2 h followed by harvesting of livers for total RNA preparation. Sirt1 and cyclophilin B mRNA levels were determined as in A. P=0.055 for WT versus HIF-1α+/−, P=0.015 for WT versus HIF-2α+/−, and P=0.004 for WT versus HIF-1α+/−/HIF-2α+/−. All comparisons made by one-tailed t-test.

**Fig. 5. HIF deficiency alters Sirt1 function during hypoxia.**

A, Endogenous HIF-1α, SIRT1, and α-tubulin as well as ectopic SIRT1 (vsv-g) protein levels were determined by immunoblot analyses in Hep3B cells after transfection with control (−) or HIF-1α (+) siRNA in the absence or presence of ectopic wild-type (W) or deacetylase defective (D) Sirt1 expression. Cells were exposed to hypoxia for 6 hours beginning 48 hours after siRNA transfection. Samples were harvested in the absence or presence of the Sirt1 inhibitors sirtinol and nicotinamide (NAM). B, Endogenous HIF-2α was immunoprecipitated from samples prepared as in A and subjected to immunoblot analysis with antibody recognizing HIF-1α or acetylated lysine. C, a model for Sirt1 activation during acute hypoxia is shown. Upon hypoxia exposure, HIF-1α and HIF-2α stabilize and bind to the HREs in the proximal Sirt1 promoter region resulting in increased Sirt1 gene expression.

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Figure 1

A
IB/WCE: acetyl-lysine
IB/WCE: HA (PPN HIF-α)

Hep3B/Sirtinol+NAM
HT1080/Sirtinol+NAM

Hypoxia (hr):
SP:PPN HIF-1α:HA
SP:PPN HIF-2α:HA

B
IB/NE: human SIRT1
IB/NE: TBP

Hep3B
HT1080

Hypoxia (hr):

C
SIRT1/Cyclophilin mRNA expression

Hep3B
HT1080

Hypoxia (hr): 0 2 4 8 16 24
**Figure 2**

**A**

Human SIRT1 Promoter

ChIP Amplicon

Wild-Type (WT): GGAAGACGTGGAA
Mutant HRE5 (mutHRE5): GGAAGACTGTA
Mutant ETS-binding sites (mutEBS): GATAGACGTGATA

**B**

Fold Induction

Hep3B

HT1080

PPN HIF-α: 1 1 2 2 1 1 2 2
SIRT1: W D W D W D W D W D

SIRT1 Prom (-354/+54): WT mutHRE5 mutEBS

**C**

Captured SIRT1 Promoter Chromatin (% input)

Hep3B

Hypoxia (hr): 0 4 8 16

Normal IgG HIF-1α IgG HIF-2α IgG
Figure 3

A

786-0

B

VHL: – +

IB: SIRT1

IB: HIF-1α

IB: HIF-2α

IB: myc (myc:VHL)

IB: α–TUBULIN

C

Hep3B

D

PPN HIF-α: – 1 2

IB: SIRT1

IB: HA (PPN HIF-α)

IB: α–TUBULIN

E

Hep3B

F

Hep3B

HIF-α siRNA: C 1 2 C 1 2

IB: SIRT1

IB: HIF-1α

IB: HIF-2α

IB: α–TUBULIN
Figure 4

A

Liver

Sirt1/Cyclophilin mRNA expression

IB: HA (HIF-α)
IB: Sirt1
IB: Tubulin

PPN HIF-α: – 1 2

C

Liver

Sirt1/Cyclophilin mRNA expression

O2 Content: 21% 6%

B

Liver

PPN HIF-α:
IB: HA (HIF-α)
IB: Sirt1
IB: Tubulin

D

Liver

IB: Sirt1
IB: Tubulin

E

Mouse Sirt1 Promoter

Captured Sirt1 chromatin (ratio to 10% input)

ChIP Amplicon

O2 Content: 21% 6%

F

Liver

Sirt1/Cyclophilin mRNA expression

Genotype: WT HIF-1α+/− HIF-2α+/− HIF-1α+/− HIF-2α+/−
**Figure 5**

**A**

Hep3B/Hypoxia

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**B**

Hep3B/Hypoxia

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**C**

Normoxia

HIF-1

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Hypoxia

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