



Published in final edited form as:

Gene. 2016 December 31; 595(2): 212–220. doi:10.1016/j.gene.2016.10.013.

Role of HIF-1 α in the Hypoxia Inducible Expression of the Thiamine Transporter, *SLC19A3*

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Abstract

Ensuring continuous intracellular supply of thiamine is essential to maintain metabolism. Cellular homeostasis requires the function of the membrane bound thiamine transporters THTR1 and THTR2. In the absence of increased dietary intake of thiamine, varying intracellular levels to meet metabolic demands during pathophysiological stressors, such as hypoxia, requires adaptive regulatory mechanisms to increase thiamine transport capacity. Previous work has established the up-regulation of *SLC19A3* (THTR2) gene expression and activity during hypoxic stress through the activity of the hypoxia inducible transcription factor 1 alpha (HIF-1 α). However, it is unknown whether HIF-1 α acts directly or indirectly to trans-activate expression of *SLC19A3*. This work utilized the breast cancer cell line BT-474 treated with 1% O₂ or a hypoxia chemical mimetic deferoxamine to determine the minimal promoter region of *SLC19A3* responsible for hypoxia responsiveness. *In silico* sequence analysis determined two contiguous hypoxia responsive elements in close proximity to the transcriptional start site of the *SLC19A3* gene. Using a HIF-1 α transcriptional factor ELISA assay, HIF-1 α was capable of binding to a dsDNA construct of the *SLC19A3* minimal promoter. Chromatin immunoprecipitation assay established that SP1 was bound to the *SLC19A3* minimal promoter region under normoxic conditions. However, HIF-1 α binding to the minimal promoter region occurred during hypoxic treatments, while no SP1 binding was observed under these conditions. This work demonstrates the direct binding and activation of *SLC19A3* expression by HIF-1 α during hypoxic stress, suggesting an important adaptive regulatory role for HIF-1 α in maintaining thiamine homeostasis.

Keywords

Hypoxia; HIF-1 α ; Thiamine; Cancer; Transporter

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1.0 Introduction

Thiamine, or vitamin B1, is an essential micronutrient that is fundamentally required to sustain the bioenergetic and anabolic needs of all cells. Due to its hydrophilicity, thiamine requires carrier-mediated transport to enter cells in order to maintain intracellular thiamine supply. Two transporters in the Solute Carrier 19 family, THTR1 and THTR2 encoded by the *SLC19A2* and *SLC19A3* genes, respectively, are primarily responsible for thiamine transport across the plasma membrane (1–3). Both THTR1 and THTR2 are ubiquitously expressed, with the highest expression of THTR1 in skeletal muscle, and the highest expression of THTR2 in the intestinal tract (2, 4). Specifically, THTR1 appears to be localized to the basolateral membrane of polarized cells and THTR2 is typically found on the apical membrane (5). Both are antiport proton-linked transporters having an affinity (*K_m*) for thiamine of ~2.5 μ M and 27nM for THTR1 and THTR2, respectively (2, 6).

The activated form of thiamine, thiamine pyrophosphate, acts as a cofactor for several key metabolic enzymes such as pyruvate dehydrogenase, transketolase and α -ketoglutarate dehydrogenase (7). The inability of mammals to synthesize thiamine *de novo* makes continuous dietary ingestion obligatory to maintain intracellular thiamine levels. Humans are highly sensitive to thiamine deficiency due to extremely limited thiamine storage ability (8). Therefore, maintenance of thiamine concentrations requires a highly regulated and adaptive process. Alterations in thiamine homeostasis can occur within diseases or physiological stresses that induce changes in cell metabolism. For example, hypoxic microenvironments within solid tumors induce a shift in metabolism towards a reliance on glycolysis to meet bioenergetic and biomass requirements within the cell (9, 10). In order to support this increased metabolic flux, a corresponding increase in nutrients and cofactors are required (11, 12). In the absence of increased dietary intake, increasing intracellular thiamine to meet metabolic demands would require adaptive regulatory mechanisms to increase transport capacity.

Recently, we have reported on the potential involvement of hypoxia inducible factor-1 alpha (HIF-1 α) in adaptively regulating thiamine transport during pathophysiological stress (13, 14). Under hypoxic conditions the expression of *SLC19A3* was adaptively up-regulated, while *SLC19A2* expression remained unchanged (13). HIF-1 α is a master stress response transcriptional factor that regulates the expression of a wide array of genes related to angiogenesis, glucose metabolism, cell survival and apoptosis (15–19). During normal oxygen conditions, HIF-1 α is degraded through hydroxylation by prolyl hydroxylases, binding of the von Hippel–Lindau protein and subsequent proteasomal degradation of the protein. In low oxygen tensions, HIF-1 α is stabilized and activated due to the inhibition of prolyl hydroxylase activity (18). HIF-1 α can then dimerize with HIF-1 β forming the HIF-1 complex, and subsequently translocate to the nucleus (18, 20, 21). Increased HIF-1 α target gene expression supports the Warburg cancer phenotype and contributes to increased tumor aggressiveness and poor patient prognosis (20, 22). Adaptively increasing thiamine transport during hypoxic stress may support the metabolic reprogramming facilitated by HIF-1 signaling. Therefore, the objective of this work was to determine whether HIF-1 α directly trans-activates *SLC19A3* expression in response to hypoxic stress.

2.0 Materials and Methods

2.1 Cell Culture

Cell culture flasks, plates and dishes were from Greiner Bio-one (Monroe, NC). Fetal bovine serum (FBS) was purchased from Seradigm (Radnor, PA). BT-474 cells (ATCC, Manassas, VA), a breast cancer cell line, were maintained in RPMI 1640 media supplemented with 10% FBS and 0.1% gentamycin (Corning, Corning, NY) in an incubator at 37°C, 5% CO₂, and 21% O₂ that is defined as normoxic conditions. Media was refreshed every 2 days, and split upon reaching approximately 70% confluency.

2.2 Hypoxic Treatments

Hypoxic conditions were achieved by treatment with the hypoxia chemical mimetic deferoxamine mesylate (Calbiochem, La Jolla, CA) (250 μM DFO) or 1% O₂ for 24, 48 or 72 hours. An incubator equipped with a ProOX oxygen controller (Biospherix, Lacona, NY) supplying nitrogen gas was used to achieve 1% O₂. Calibration of the oxygen controller was performed weekly, while fyrite gas analyzer was used to check oxygen levels daily (Bacharach INC, New Kensington, PA). Media was pre-equilibrated at 1% O₂ for a minimum of 24h before use. Cells were refreshed with DFO containing media or 1% O₂ equilibrated media every 24h during treatments.

2.3 Promoter Constructs

In silico sequence analysis revealed seven and four putative HREs within the *SLC19A2* and *SLC19A3* promoter regions, respectively, with the sequence 5'-R-CGTG-3' upstream of the transcription start site (+1) (Fig 1A). The putative HREs were located at -2051, -1879, -1732, -1276, -137 and -111 in the *SLC19A2* promoter region, and -1336, -908, -55 and -47 for *SLC19A3*. Promoter constructs for *SLC19A2* and *SLC19A3* were created by cloning the promoter region of the gene of interest from genomic DNA (Clontech, Mountain View, CA) with Pfu polymerase (Agilent Technologies, Santa Clara, CA). Primers used for cloning were designed corresponding to known sequences of *SLC19A2* and *SLC19A3* promoters (Table 1) (23, 24). In order to establish which HRE regions of the *SLC19A3* promoter are involved in adaptive regulation, 5' truncations of the full *SLC19A3* promoter were made as depicted in Fig 1B. These deletion constructs were generated by PCR using Pfu polymerase, genomic DNA, and the oligonucleotide primers indicated in Table 1. For the full promoter (-1957/+59), -970/+59, -473/+59 and -32/+59 the reverse primer was *SLC19A3*+59 and an NheI site was added to the 5' end of the primer. All forward primers for the constructs had a KpnI site added. The PCR products and pGL3 basic plasmid were restriction digested with NheI and KpnI (Promega, Madison, WI) at 37°C for 1 h followed by 65°C for 15 min. Digested fragments and plasmids were ligated using T4 DNA ligase (Promega, Madison, WI). Sequences were verified through the University of Georgia Genomics sequencing facility. An HRE plasmid construct containing three subcloned copies of (5'-GTGACTACCGTGCTGCCTAG-3') the inducible nitric oxide synthase promoter (pGL3-HRE) was generously donated by Dr. Giovanni Melillo and used as a positive control (25).

2.4 Site-directed Mutagenesis

To further investigate the role of each putative HRE in *SLC19A3* adaptive regulation, site-directed mutagenesis on the full (-1957/+59) *SLC19A3* pGL3 construct was performed. Mutation constructs were prepared following the manufacturer's protocol for the Quikchange site-directed mutagenesis II kit (Agilent Technologies, Santa Clara, CA). Briefly, primers were designed to include the desired mutation flanked by an unmodified portion of the HRE nucleotide sequence (Table 2). After mutant strand synthesis, digestion of the parental methylated DNA was performed by adding 1 μ L of the Dpn I restriction enzyme (10 U/ μ L) directly to the amplification reaction. The reaction mixture was then incubated at 37°C for 1 h to digest the parental (i.e., the non-mutated) supercoiled dsDNA.

XL1-Blue supercompetent cells were gently thawed on ice and 50 μ L was aliquoted into a prechilled 14 mL BD Falcon polypropylene round-bottom tube. 1 μ L of the Dpn I treated DNA was transferred to the supercompetent cells. The transformation reaction was gently swirled and incubated on ice for 30 min, heat pulsed at 42°C for 45 sec then placed back on ice for 2 min. 0.5 ml of NZY+ broth (preheated to 42°C) was added to the supercompetent cells and incubated at 37°C for 1 h with shaking at 225–250 rpm. Plasmids were harvested using E.Z.N.A.® Fastfilter Plasmid Midi Kit (Omega Biotek, Norcross, GA). Sequences were verified through the University of Georgia Genomics sequencing facility.

2.5 Luciferase assay

BT-474 cells were reverse transfected using Lipofectamine LTX (Life Technologies, Carlsbad, CA) with pGL3-promoter constructs and the pRL-SV40 vector (Promega, Madison, WI) encoding the Renilla luciferase gene used as a control for transfection efficiency. Transfection complexes were prepared as per manufacturer's protocol with a reagent (μ L):DNA(μ g) ratio of 3.6:1. The firefly luciferase plasmid:renilla ratio was 25:1. 250 μ L of transfection complex was added to 35 mm dishes followed by 100,000 cells/cm² in 2 mL medium. Cells were incubated with transfection complex for 6 h at which time media was changed and cells allowed to recover for 24h. After treatment with 1% O₂ for 48h, promoter activity of transfected plasmid constructs was quantified with the Dual Luciferase Reporter® assay (Promega, Madison, WI) following manufacturer's protocol using a Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT). Results were normalized to total protein measured using the BCA Protein Assay (Thermo Scientific, Rockford, IL). The results are represented as a fold change of the ratio of firefly to Renilla luminescence in hypoxic to normoxic values.

2.6 Expression of Thiamine Transporters and Transcriptional Factors

To assess THTR1 and THTR2 expression after hypoxic treatment for up to 72h, BT-474 cells were harvested as whole cell lysates (WCL). To obtain WCL, treated cells were washed in ice-cold phosphate buffered saline (PBS) and lysed using WCL buffer (1% Nonidet P-40 (NP40), 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.01% sodium azide, 50mM tris, 250mM NaCl, and 1mM ethylenediaminetetraacetic acid (EDTA) at pH=8.5) supplemented with protease and phosphatase inhibitors and phenylmethanesulfonyl fluoride (Calbiochem, La Jolla, CA). Lysates were collected and centrifuged at 17,000xg using a Microfuge 22R Centrifuge (Beckman Coulter, Brea, CA) for

15 min at 4°C and the supernatant was collected. Expression of the established HIF-1 α target gene lactate dehydrogenase A (LDHA), was utilized as a marker for functionally active HIF-1 α (26). For transcriptional factor expression of SP1 and HIF-1 α , BT-474 nuclear and WCL were obtained after hypoxic treatment for up to 72h. To harvest nuclear lysates, cells were incubated in trypsin/EDTA, collected and centrifuged at 500xg using a Microfuge 22R Centrifuge for 5 min at 4°C, washed in PBS, and centrifuged again. Cell pellets were then lysed in 1mL cytoplasmic extraction buffer (10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM ethylene glycol tetraacetic acid (EGTA)), vortexed, and left on ice for 10 min. 10% NP40 was added to each sample at a ratio of 62.5 μ L/1mL of cytoplasmic extraction buffer, sample was then vortexed and left on ice for one min. Nuclei were pelleted by centrifugation at 17,000xg in a Microfuge 22R Centrifuge for 10 min at 4°C. Supernatant was discarded, and nuclei were lysed in 200 μ L nuclei extraction buffer (20mM HEPES, 0.4M NaCl, 1mM EDTA, 1mM EGTA) and left on ice for 40 min, with vortexing every 10 min. Samples were then centrifuged at 17,000xg in a Microfuge 22R Centrifuge for 10 min at 4°C and supernatant was collected as the nuclear lysate. Protein was quantified using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) following the manufacturer's instructions. Thiamine pyrophosphokinase 1 (TPK1), a protein located exclusively in the cytoplasm, was utilized as a marker for cytoplasmic contamination of nuclear samples (27).

WCL (50 μ g) or nuclear lysates (25 μ g) of each sample were resolved by electrophoresis on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% non-fat milk in tris buffered saline-tween 20 (TBS-T) for 1 h at 4°C. The membrane was immunoblotted for HIF-1 α , LDHA, TPK1, β -actin, p84 (Genetex, Irvine, CA), *SLC19A3* (Sigma Aldrich, St. Louis, MO), THTR1 (Alpha Diagnostics, San Antonio, TX) or SP1 (SantaCruz Biotechnology, Dallas, TX) overnight at 4°C. Blots were washed 3 times for 10 min each in TBS-T, and then immunoblotted with 1:20,000 goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Millipore, Billerica, MA) or 1:20,000 goat anti-rabbit HRP-conjugated secondary antibody (Bethyl Laboratories, Montgomery, TX) for 1 h at room temperature. Blots were visualized with Supersignal West Pico (Thermo Scientific, Rockford, IL) and captured with a Fluorchem SP digital imager (Alpha Innotech, San Leandro, CA). Densitometry was performed using Fluorchem SP software.

2.7 HIF-1 α Transcription Factor Binding Assay

An ELISA-based HIF-1 α transcription factor assay (Abcam, Cambridge, MA) was used as a qualitative approach to determine HIF-1 α binding to the minimal promoter region of *SLC19A3*. The more available HIF-1 α present in the sample, the greater HIF-1 α binding to the R-CGTG HIF-1 α consensus sequence bound to the plate surface. With the addition of competitor R-CGTG containing dsDNA sequences that are not adhered to the plate, we would anticipate a decrease in the absorbance reading compared to a non-specific control. This assay was utilized synonymously for an EMSA to show specificity of transcription factor binding to the promoter region of a gene. The addition of a known dsDNA sequence to the initial binding reaction demonstrates competition for binding with the immobilized DNA, analogous to the shift seen in an EMSA. Addition of a mutated version of the promoter region demonstrates specific binding to the competitor oligonucleotide at the

mutated portion of the sequence. In this way, we can obtain similar information as with an EMSA.

Within the *SLC19A3* minimal promoter region from -473 to +59 bp, two putative HREs were identified as displayed in Fig 1A. A 40bp region surrounding these HREs was synthesized as an oligonucleotide construct (Integrated DNA Technologies, Coralville, IA) and used as a specific competitor. This construct will be referred to as *SLC19A3* Competitor (Sense 5'-ATT CGC ATA TGC AAA GCG TGG GGG CGT GGC CCC GGG CTCC-3'). To produce the *SLC19A3* Mutant (Sense 5'-ATT CGC ATA TGC AAA GAA AAG GGG AAA AGC CCC GGG CTCC-3'), the two CGTG HIF-1 α consensus sequences were mutated to AAAA to control for non-specific binding. The single-stranded sense and complementary antisense strand were annealed starting at a temperature of 95°C and reduced by 1° each cycle, for 70 cycles in 10mM Tris, 1mM EDTA, 50mM NaCl buffer. dsDNA of a random R-CGTG HIF-1 α consensus sequence was also supplied by the manufacturer and was used as a positive control for competition.

BT-474 cells treated with 1% O₂ for 24h were harvested as nuclear lysates as described above. Nuclear lysates (10 μ g) were diluted in supplied complete transcription factor binding assay buffer, with or without addition of oligonucleotide competitors. Oligonucleotides used were 25 μ M of the *SLC19A3* competitor, *SLC19A3* mutant, and the competitor provided by the manufacturer. Samples were incubated for 20 min at room temperature before being added to the plate in triplicate. Plates were incubated at 4°C overnight (~14h), then washed 5 times with wash buffer and incubated for 1h at room temperature with a rabbit anti-HIF-1 α antibody supplied by the manufacturer. Plates were washed 5 times with wash buffer and then incubated with a goat anti-rabbit HRP conjugated secondary antibody that was supplied by the manufacturer for 1h at room temperature. Developing solution was added, and plates were incubated for 30 min at room temperature with gentle shaking. Finally, transcription factor stop solution was added, and the absorbance at 450nm was measured using a SpectraMax M2 spectrophotometer (Molecular Devices; Sunnyvale, CA).

2.8 Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed using the ChIP-IT Express Enzymatic kit following the manufacturer's instructions (Active Motif, Carlsbad, CA). Briefly, BT-474 cells were grown to approximately 80% confluency in 15cm dishes, and treated with 250 μ M DFO or 1% O₂ for 24h, as described above. Cells were fixed for 10 min in formaldehyde (VWR, Radnor, PA), lysed, and then dounced on ice to release nuclei. Nuclei were collected and digested to release chromatin then enzymatically sheared for 30 min. A 50 μ L aliquot was used to assess DNA concentrations following the manufacturer's protocol for ethanol precipitation, and then quantified using a Nanodrop 2000c Spectrophotometer (Thermo Scientific, Waltham, MA). A 10 μ L aliquot of sheared chromatin was set aside as input DNA for a positive control, which did not undergo any immunoprecipitation reactions. 2 μ g of each chromatin sample was added along with 1 μ g of the desired antibody for the immunoprecipitation reaction. Antibodies used were directed against HIF-1 α (Bethyl Laboratories, Montgomery, TX), SP1 (Genetex, Irvine, CA) and a nonspecific IgG (SantaCruz Biotechnology, Dallas, TX). These reactions were incubated overnight (~14h) at 4°C on a rotator and chromatin was

eluted and processed following manufacturer's instructions. Processed chromatin was used as a template during end point PCR analysis, along with the input sample as a positive control. *SLC19A3* HRE primers (Sense: 5'- AAA CGA TCG CTG TTG GAT TC-3'; Antisense: 5'- ATC CAG GCG CTC TTG GTG-3'; Amplicon: 250bp) are directed against the *SLC19A3* minimal promoter region. Econotaq Plus Green Master Mix reagents (Lucigen, Middleton, WI) were used to create the PCR reactions, which were run with an annealing temperature of 59°C for 40 cycles. A 2.5% agarose gel was run at 100V for 1h and PCR products were visualized using Fluorchem SP digital imager (Alpha Innotech, San Leandro, CA).

2.9 Statistical Analysis

All experiments were performed with a minimum of three independent experiments. Statistical significance was evaluated between groups using either Student's t-test, one-way analysis of variance with Tukey's post hoc test, or a two-way analysis of variance with Bonferroni's multiple comparison's test with a significance level of $p < 0.05$ using Graphpad Prism 6® (GraphPad Software; La Jolla, CA).

3.0 Results

3.1 Changes in protein expression after hypoxia exposure

When BT-474 cells were exposed to hypoxic conditions for 24, 48 and 72 h, there was an ~35 fold increase in total HIF-1 α protein levels after 24h DFO and an ~5 fold increase after 1% O₂ treatment for 72h (Fig 2). Protein expression of the established HIF-1 α target gene LDHA significantly increased ~2.4 fold after 72h DFO exposure and ~2.5 fold after 1% O₂ exposure demonstrating functionally active HIF-1 α during hypoxic treatments (Fig 2). THTR1 expression did not change after exposure to either DFO or 1% O₂ (Fig 2). In contrast, there was an approximately 2 fold statistically significant increase in THTR2 expression after DFO exposure, and ~1.5 fold increase in protein expression after 1% O₂ exposure up to 72h (Fig 2). Total SP1 protein levels did not change with either hypoxic treatment (Fig 2).

3.2 *SLC19A2* and *SLC19A3* promoter activity after 1% O₂ exposure

Luciferase reporter assays were utilized to investigate the hypoxia responsiveness of the *SLC19A2* and *SLC19A3* promoters. A significant increase in luciferase activity was observed using the positive control pGL3-HRE plasmid compared to the empty pGL3 basic vector demonstrating that 1% O₂ exposure for 48h is sufficient to induce HIF-1 α activity (Fig 3A). After 48h exposure to hypoxia the full length *SLC19A3* promoter showed an approximately 3.0 fold increase in luciferase expression while there was no change (~1 fold) in *SLC19A2* promoter activity (Fig 3B).

The deletion constructs of the *SLC19A3* promoter represented in Fig 1B were created to identify which of the putative HREs contribute to the hypoxic responsiveness. Truncation of the promoter to lengths of 1,029 bp (-970/+59) and 532 bp (-473/+59) resulted in no significant change in promoter activity after 48h 1% O₂ exposure compared to the full *SLC19A3* promoter (-1957/+59) (Fig 3B). Truncation of the promoter to 91 bp (-32/+59

construct), which removes all of the putative HREs before the transcriptional start site resulted in a significant decrease in the promoter activity to approximately 1.5 fold after hypoxia exposure.

To further investigate which putative HREs play a role in the hypoxia responsiveness of *SLC19A3*, we created site-directed mutants. Mutations in the full-length promoter at -1336, -908, or -55 demonstrated no significant change in the hypoxia responsiveness of the *SLC19A3* promoter (Fig 3C). Since HRE-47 overlaps with a reported SP1 binding site important for *SLC19A3* basal expression in the minimal promoter region, two mutations were made for this HRE (Fig 3D) (28). The first mutation -47a was of the CGTG sequence of the HRE to AATG, which also mutated the SP1 binding site. The second mutation -47b was of the CGTG sequence of the HRE to CGAA, leaving the SP1 binding site unmodified. Compared to the full *SLC19A3* promoter, there was no significant change in promoter activity of either HRE -47 mutation after 1% O₂ exposure for 48h. When both -55 and -47 were mutated, there was a significant loss in the hypoxia responsiveness with a fold change of ~1 after hypoxia exposure compared to normoxia (Fig 3C).

3.3 HIF-1 α Binding to the *SLC19A3* Promoter

Normoxic control BT-474 nuclear lysates containing minimal activated HIF-1 α demonstrated no significant change in absorbance after addition of competitors (Fig 4). In contrast, nuclear lysates from BT-474 cells treated with 1% O₂ for 24h have a significantly increased amount of activated HIF-1 α protein as compared to the normoxic control sample. Addition of a specific competitor supplied by the kit (Supplied Competitor), as well as the *SLC19A3* promoter construct (*SLC19A3* Competitor) resulted in a statistically significant decrease in absorbance (Fig 4). Addition of a mutated form of the *SLC19A3* promoter (*SLC19A3* Mutant) resulted in a statistically significant increase in absorbance as compared to the Supplied Competitor and *SLC19A3* Competitor (Fig 4).

3.4 Nuclear localization of transcriptional factors after hypoxia exposure

To investigate the effects of hypoxia on the nuclear localization of SP1 and HIF-1 α , Western blots were performed on nuclear fractions (Fig 5A–D) of BT-474 cells treated with 1% O₂ or 250 μ M DFO for up to 72h. HIF-1 α nuclear localization significantly increased up to ~11 fold after 1% O₂ exposure and ~8 fold after DFO exposure (Fig 5A–D). No change in nuclear localization for SP1 was found up to 72 h of 1% O₂ or DFO treatment compared to normoxia (Fig 5A–D). No TPK1 expression was observed in nuclear samples, as compared to the WCL control demonstrating no cytoplasmic contamination within the samples (Fig S1).

3.5 *In vitro* Binding of HIF-1 α to the *SLC19A3* promoter

Chromatin immunoprecipitation (ChIP) was used to detect HIF-1 α bound to the *SLC19A3* promoter in BT-474 cells treated with either 250 μ M DFO or 1% O₂ for 24h. An amplicon size of 250 bp using sheared chromatin that was not immunoprecipitated (input) was used as a positive control for *SLC19A3* minimal promoter detection in the sample (Fig 5E). Hypoxia treated samples (1% O₂ and DFO) immunoprecipitated with an antibody directed against HIF-1 α detected a band at 250 bp. No detectable band appeared at 250bp with a nonspecific

IgG antibody in all groups, or with the HIF-1 α antibody in control normoxia samples. In contrast, the *SLC19A3* promoter was detected with SP1 pulldown in normoxia control but not in 1% O₂ or 250 μ M DFO treatments (Fig 5E).

4.0 Discussion

Previously, we have established that the high affinity thiamine transporter, THTR2 encoded by the *SLC19A3* gene was transcriptionally up-regulated after hypoxic stress (13). The increase in *SLC19A3* gene expression correlated with an increase in overall thiamine transport, suggesting an important role for adaptively increasing intracellular thiamine levels during hypoxia (13). The role of HIF-1 α in mediating *SLC19A3* expression was previously demonstrated through the reduction in *SLC19A3* expression using the HIF-1 α inhibitor compound YC1, a HIF-1 α dominant negative plasmid, and shRNA knockdown of HIF-1 α (13, 29). However, these findings do not reveal if HIF-1 α is directly or indirectly trans-activating *SLC19A3* expression during hypoxia. Using a HIF-1 α transcription factor assay and chromatin immunoprecipitation we have confirmed for the first time that HIF-1 α directly binds to the *SLC19A3* promoter to adaptively induce expression during hypoxic stress.

Interestingly, although the *SLC19A2* promoter contains 7 HREs, no change in mRNA expression was previously reported in response to hypoxic stress (13). The lack of hypoxia responsiveness of *SLC19A2* was further established with no significant change in luciferase activity for the cloned *SLC19A2* full promoter (Fig 3B). These results, combined with the absence of a significant change in protein expression (Fig 2) demonstrate that *SLC19A2* expression is not adaptively regulated in response to hypoxic conditions. The finding of HRE's within the *SLC19A2* promoter may be coincidental since the sequence of the HIF-1 α responsive element contains only 4 nucleotides. However, genome-wide analysis has revealed that less than 1% of potential HREs within the genome are actually HIF-1 α targets (30). On an evolutionary perspective, adaptability in the regulation of the high affinity thiamine transporter *SLC19A3* would be consistent with maximizing cellular transport under thiamine limited conditions, while the low affinity *SLC19A2* may provide for basal transport and not require adaptive regulatory processes. Although the mechanistic reason behind a lack of response by the HREs found in the *SLC19A2* promoter is unclear, the sequence and spacing of HREs may play a key functional role. HIF-1 α target genes typically contain an HRE sequence (R-CGTG) with either a direct repeat or a hypoxia ancillary sequence (HAS) consisting of an inverted imperfect repeat (30–32). It is possible that the HREs may be too far apart to be bound by HIF-1 α in the *SLC19A2* promoter, as the closest HREs are 22bp apart. Moreover, it was previously determined that HREs bound by HIF-1 α tend to be located proximally to the transcriptional start site of target genes (30). The closest HRE to the transcriptional start site in the *SLC19A2* promoter is 111bp, while the closest HRE in *SLC19A3* is only 47bp away (Fig 1A). Therefore, the spatial arrangement of the HREs in the *SLC19A2* gene may be a major factor in the lack of hypoxic responsiveness.

Using promoter truncations, hypoxia mediated induction of *SLC19A3* was attributed to a region containing two direct HRE repeats located at –55 and –47 upstream from the

transcription start site. Although this contiguous arrangement is consistent with the sequence and spacing observed for other HIF-1 α regulated genes, both HREs were capable of inducing promoter activity independent of each other. At present it is unclear why there appears to be a redundancy in HRE binding sites. Upon further examination, two HASs with the sequence CAGGC forming an imperfect inverted repeat of the GCGTG HRE can be identified in the minimal promoter region beginning at location -26 (Fig 3D). However, the spacing arrangement between the HREs and HASs do not appear to be consistent with several other HIF-1 α genes of approximately 9bp apart (31, 32). It is plausible that the spacing between the HASs and HREs may be distinct in allowing functional activation compared to other HIF-1 α regulated genes. Further work is necessary to evaluate the potential role of these postulated cis-enhancer elements in the adaptive regulation of *SLC19A3* during hypoxia.

The HRE located at -47 that facilitated hypoxic-responsive expression of *SLC19A3* also overlaps a GC rich region known to be a target for the transcription factor SP1 (Fig 3D) (33). Nabokina *et al.* identified that SP1 binding to this GC rich region within a similar minimal promoter region in the *SLC19A3* promoter was responsible for basal gene expression under normal physiological conditions (28). Loss of SP1 expression in diseases such as alcoholism and diabetes has been associated with decreased *SLC19A3* expression (34–36). In hypoxia, an increase in SP1 expression and nuclear localization has been reported that may be accountable for increasing *SLC19A3* expression (37–39). SP1 binding to the *SLC19A3* promoter was observed under normal oxygen conditions consistent with the known basal regulatory role of SP1 in the expression of *SLC19A3* (28). However, during hypoxic stress HIF-1 α binding was detected to the *SLC19A3* minimal promoter region with no measurable SP1 binding (Fig 5E). No significant change in either total or nuclear SP1 after hypoxic treatments to BT-474 cells was observed that could account for the loss of SP1 binding to the *SLC19A3* minimal promoter region. Overall this suggests that adaptive regulation of *SLC19A3* during hypoxic stress does not require SP1 binding and that adaptive regulation may have distinct transcriptional machinery from basal regulation. Further work is needed to understand how this regulatory switch from basal regulation mediated by SP1 to HIF-1 α controlled adaptive regulation occurs.

In cancer, HIF-1 α activation is known to support a metabolic shift within cells towards a glycolytic phenotype, supporting cell survival during low oxygen stress (20, 22, 40). Since thiamine-dependent enzymes are central in cell metabolism, it is possible that thiamine homeostasis is bolstered in hypoxia to support this metabolic phenotype. For instance, the activity of the thiamine-dependent enzyme transketolase is significantly increased in cancer cells (41, 42). This increased activity leads to a greater production of ribose-5-phosphate to support nucleotide synthesis, as well glyceraldehyde-3-phosphate and fructose-6-phosphate to increase the rate of glycolysis (41, 43). Other studies have shown that various transketolase isoforms are over-expressed in a wide range of cancers, contributing to tumor cell proliferation (42, 44, 45). Diffusional barriers to nutrient delivery within hypoxic tumor microenvironments also exist that may limit supply to cancer cells distal from blood vessels (46). Thus, adaptively regulating the expression of the high affinity thiamine transporter THTR2 may provide an efficient mechanism to maximize transport of limited extracellular thiamine into the cancer cell.

Adaptive changes in thiamine homeostasis and requirements for thiamine may also be essential for non-malignant cell survival during physiological and nutritional stress. Hypoxia mediated up-regulation of THTR2 protein was also observed in human non-neoplastic fibroblasts, suggesting that an adaptive regulatory mechanism also functions within normal tissue (47). Studies in neonatal rat cardiomyocytes have demonstrated that thiamine supplementation was protective against hypoxia-induced apoptosis, suggesting a critical role for thiamine homeostasis supporting cell survival during hypoxic stress (48). An adaptive up-regulation of *SLC19A3* expression has also been demonstrated during thiamine deficient conditions to increase transport capacity and cellular availability of thiamine (29, 49). Interestingly, the increase in *SLC19A3* during thiamine deficiency was attenuated by an inhibitor and a dominant negative construct of HIF-1 α (29). Aside from hypoxia, HIF-1 α can also be stabilized in normal oxygen conditions through a buildup of the metabolic intermediates pyruvate and lactate, termed pseudohypoxia (50). A major metabolic consequence of thiamine deficiency is a reduction in enzyme activity of pyruvate dehydrogenase complex (PDH) resulting in excess pyruvate and lactate production (51, 52). An increase in both pyruvate and lactate was observed during thiamine deficiency with subsequent stabilization and activation of HIF-1 α (29). This suggests that thiamine deficiency and hypoxic stress may exhibit a common adaptive regulatory mechanism centralizing through HIF-1 α activation. Further studies are warranted to understand the role of HIF-1 α in adaptive thiamine homeostasis during malignant and non-malignant conditions associated with thiamine deficiency and hypoxic stress such as diabetes, Wernicke-Korsakoff Syndrome, and cerebrovascular ischemic stroke (53–57).

4.1 Conclusions

In conclusion, this work demonstrates that HIF-1 α directly binds to the *SLC19A3* promoter to adaptively up-regulate the expression of the high affinity thiamine transporter THTR2 during hypoxic stress. Furthermore, the up-regulation of THTR2 occurs independent of the known basal regulatory role of SP1. Overall these findings suggest an important function of adaptive thiamine transport in pathophysiological conditions involving HIF-1 α signaling and hypoxic stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research reported in this publication was supported by the National Institute On Alcohol Abuse And Alcoholism of the National Institutes of Health under award number R21AA021948.

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- The high affinity thiamine transporter THTR2 is adaptively regulated by HIF-1 α in hypoxia
- Adaptive regulation of THTR2 is independent of the basal regulatory role of SP1

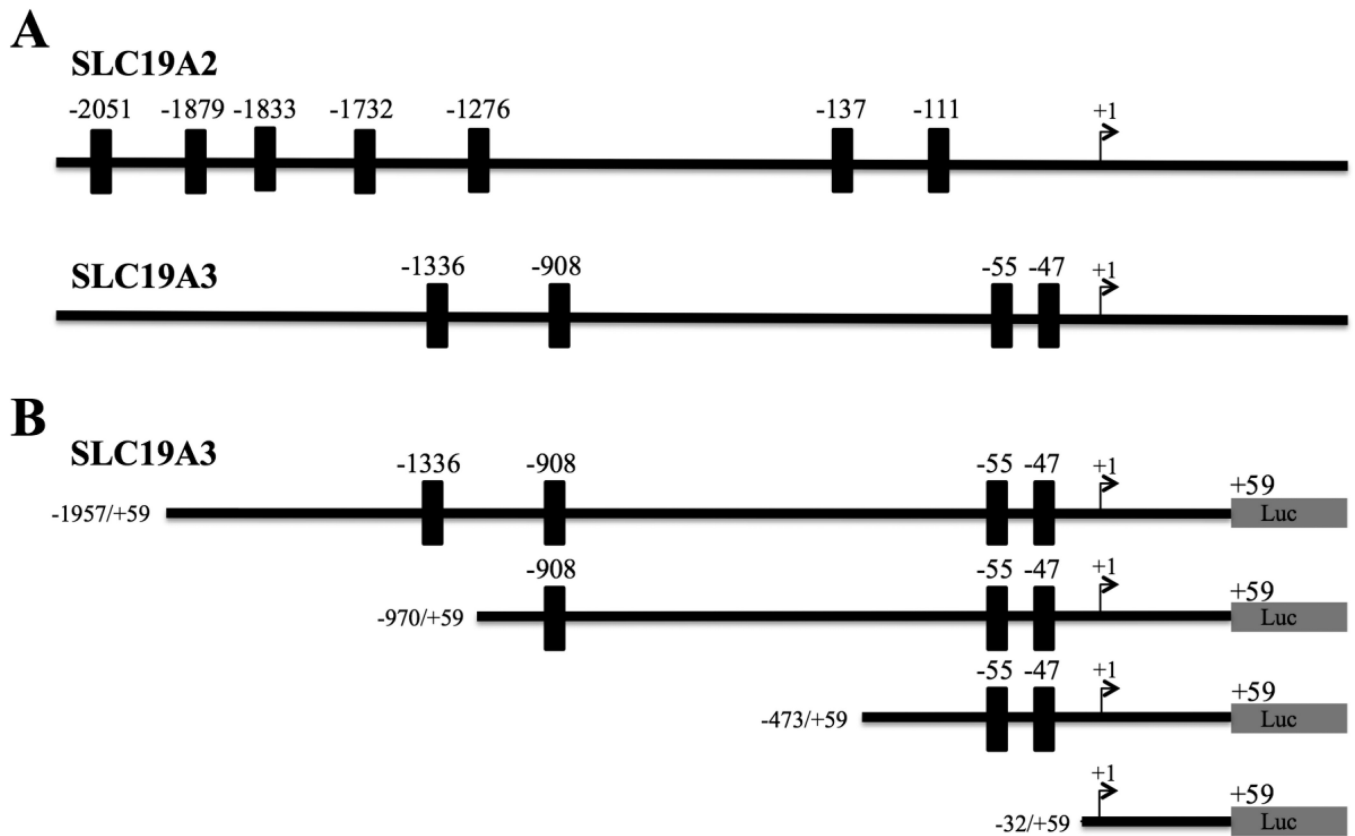


Fig 1. *SLC19A2* and *SLC19A3* full promoters and promoter constructs

A) *In silico* analysis of the *SLC19A2* and *SLC19A3* promoter regions indicating HIF-1 α responsive elements (HRE). The locations of the putative HRE sites (5'-R-CGTG-3') are indicated by black boxes. Designated numbers are relative to the transcriptional start site (+1). B) *SLC19A3* promoter and the series of 5'-deletion constructs subcloned into the firefly luciferase reporter gene vector (pGL3).

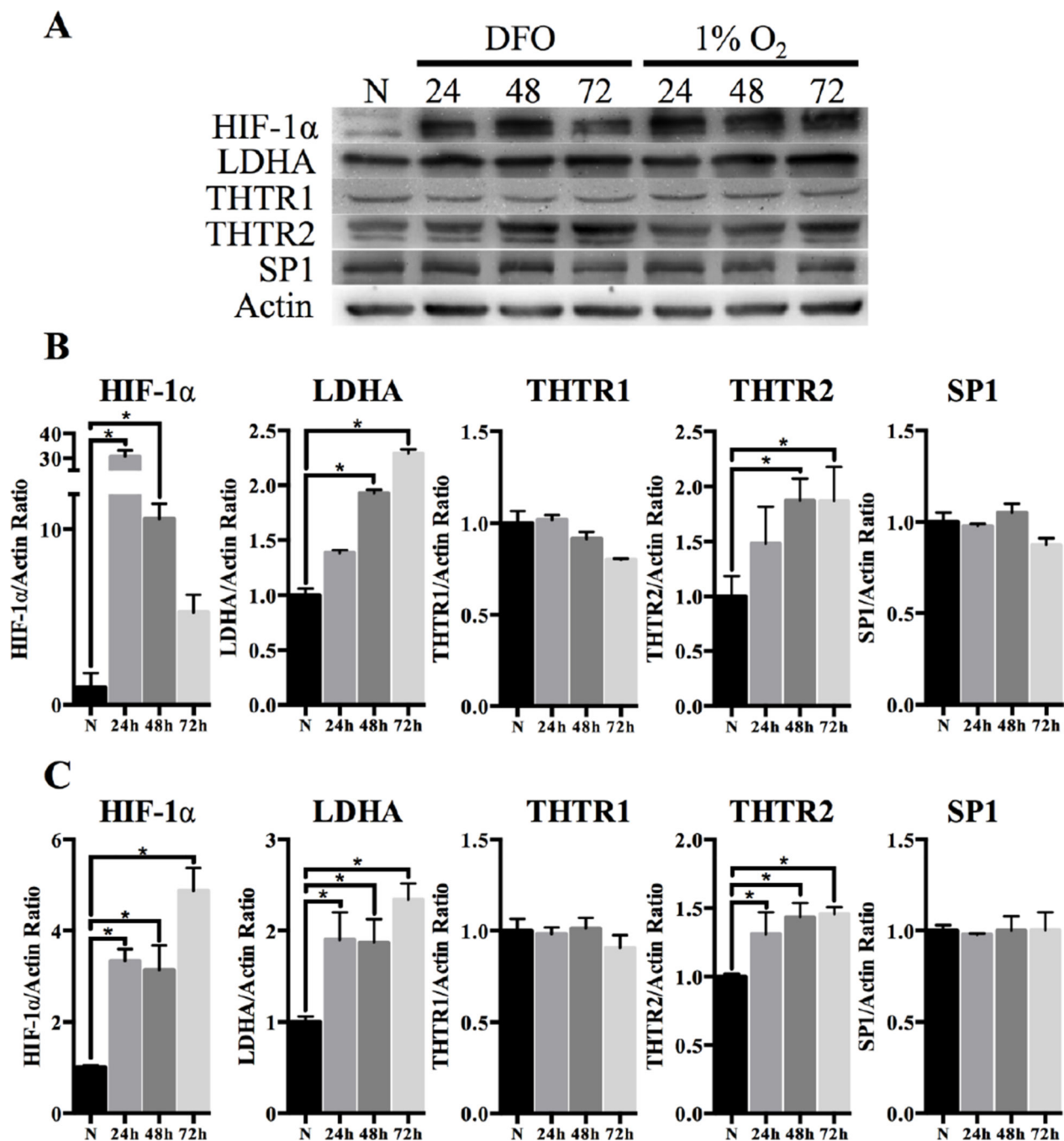


Fig 2. Changes in protein expression after hypoxic treatments

(A) Representative Western blot for HIF-1 α , LDHA, THTR1, THTR2 and SP1 expression in BT-474 whole cell lysates after 250 μ M DFO and 1% O₂ exposure for 24, 48, and 72h compared to normoxic (N) control. Actin was used as a loading control. Densitometry of protein expression after (B) DFO or (C) 1% O₂ treatment with n=7 independent experiments for HIF-1 α and n=3 independent experiments for all other proteins. (*) Represents a statistically significant difference of p<0.05 based on the results of a one-way ANOVA with Tukey's post-hoc test.

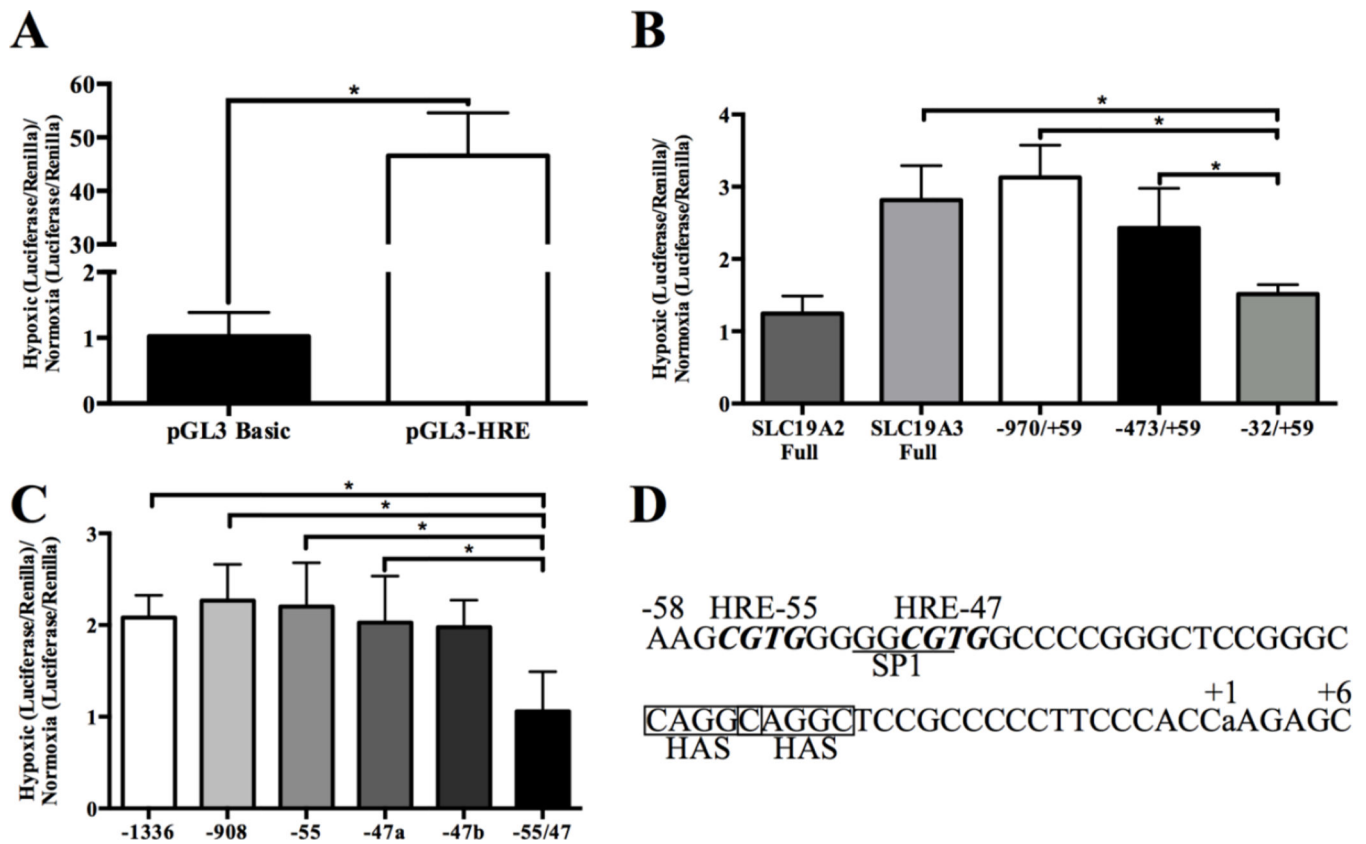


Fig 3. Promoter activity after hypoxia exposure

Transfected BT-474 cells were exposed to 1% O₂ for 48h or to normoxia. The dual luciferase assay was used to quantify the level of promoter activity and the results are reported as the fold change +/- standard deviation of the hypoxia to normoxia luciferase activity ratio with n=5 independent experiments. (A) Empty pGL3 Basic and pGL3-HRE as positive control. (B) Full *SLC19A2*, full *SLC19A3* and *SLC19A3* deletion constructs. (C) Promoter activity of the full length *SLC19A3* mutation promoter constructs. (D) *SLC19A3* minimal promoter region. Locations of HREs are indicated by bold and italics while the SP1 binding element is underlined. The location of potential HASs are indicated with a box. The +1 indicates the location of the transcriptional start site. (*) Represents a statistically significant difference of p<0.05 based on the results of a Student's T-test (A) or a one-way ANOVA (B and C) with Tukey's post-hoc test.

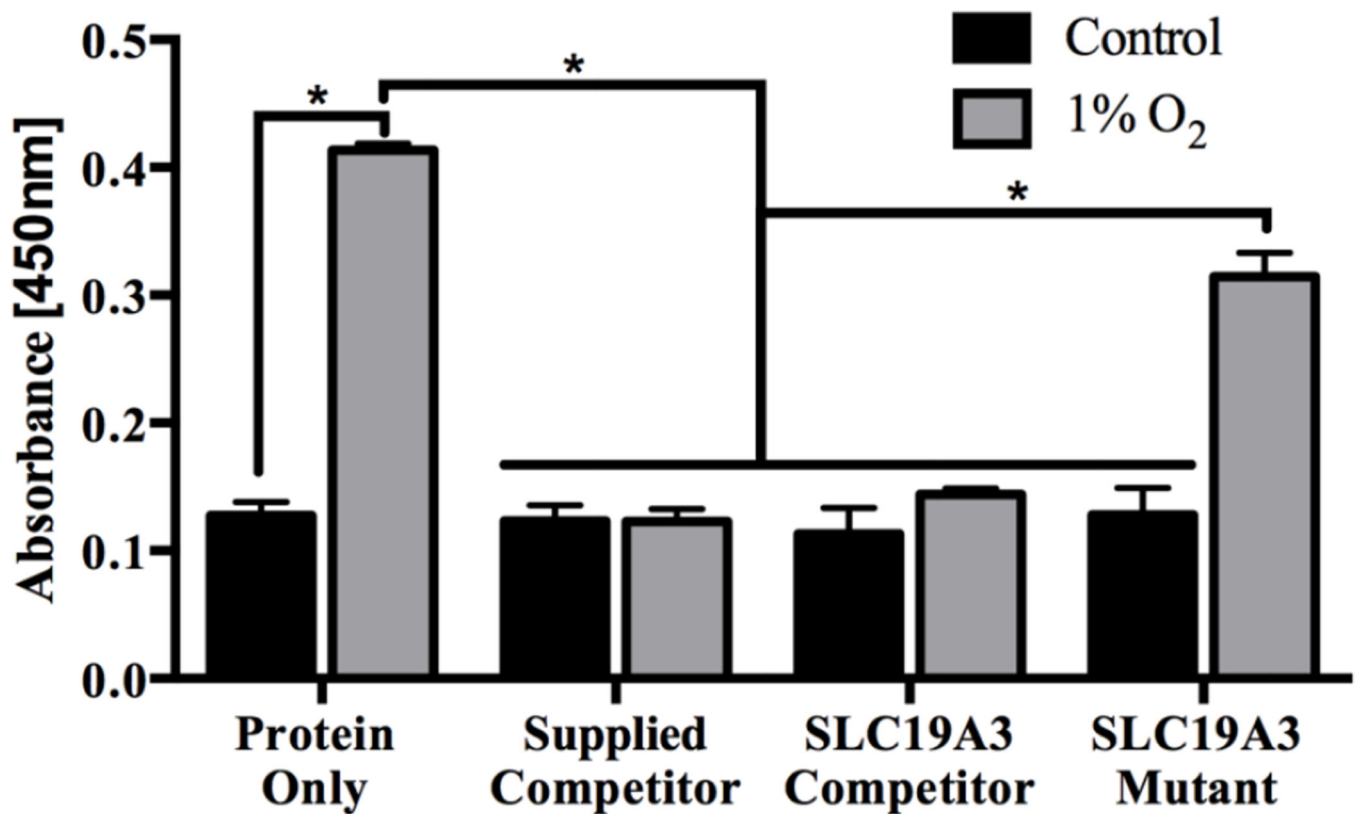


Fig 4. HIF-1 α transcription factor binding assay

BT-474 nuclear lysates treated with 1% O₂ for 24h or untreated normoxic controls are represented in the protein only group. Specific competition was demonstrated by addition of the manufacturer's supplied competitor containing a nonspecific R-CGTG sequence as well as the *SLC19A3* promoter construct containing two putative HREs. The *SLC19A3* mutant construct had each CGTG sequence mutated to AAAA. Each oligonucleotide construct was added at a concentration of 25 μ M, and incubated with nuclear lysates for 20 min before determination of HIF-1 α binding. HIF-1 α binding is represented by absorbance at 450nm +/- standard deviation of n=3 independent experiments. (*) Represents a statistically significant difference of p<0.05 as determined by a two-way ANOVA with Bonferroni's multiple comparison's test.

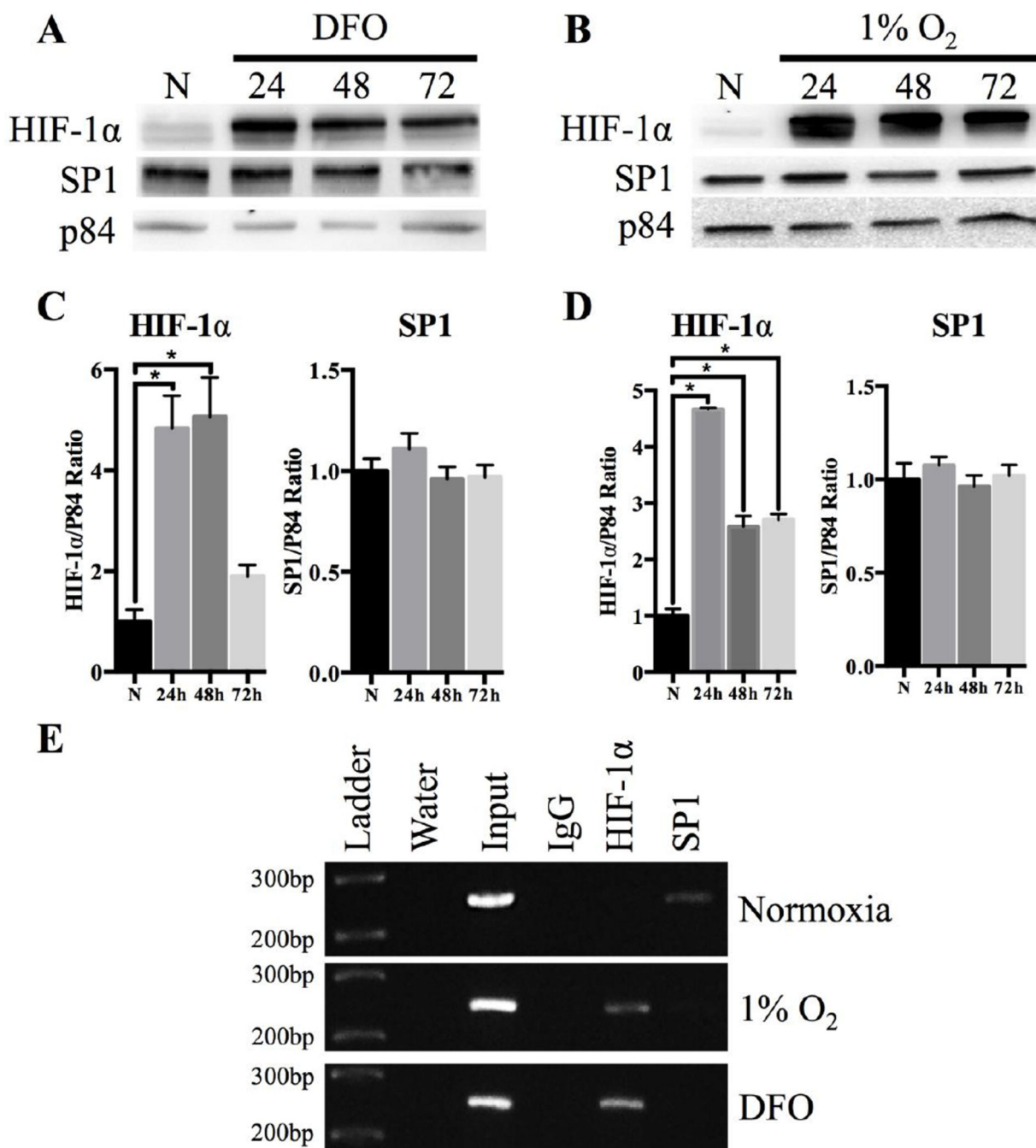


Fig 5. Nuclear localization of transcription factors after hypoxia treatment and Chromatin Immunoprecipitation Assay

Representative Western blot of HIF-1α and SP1 expression in nuclear lysates after (A) 250μM DFO and (B) 1% O₂ exposure for 24, 48, and 72h. P84 was used for loading control. Densitometry of transcription factor protein expression after (C) DFO or (D) 1% O₂ exposure up to 72 h. Data are presented with n=5 independent experiments. (*) Represents a statistically significant difference of p<0.05 as determined by a one-way ANOVA with Tukey's post-hoc test. (E) Chromatin Immunoprecipitation assay for BT-474 cells treated with hypoxia (1% O₂ or 250μM DFO) for 24h and untreated normoxic controls. Water only

reactions containing no chromatin and input reactions were used as negative and positive controls, respectively. HIF-1 α and SP1 reactions represent chromatin immunoprecipitated, while a random IgG was used as a negative control. The minimal promoter region of the *SLC19A3* promoter was signified by an amplicon of 250bp.

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Table 1
***SLC19A2* and *SLC19A3* primers**

Oligonucleotide primers used to isolate the *SLC19A2* full promoter, *SLC19A3* full promoter, and *SLC19A3* deletion constructs. KpnI sites are underlined and NheI are italicized.

Primer Name	Sequence
<i>SLC19A2</i>	Sense: 5'-TAT <u>CGG TAC CTA</u> TGT AGC CCC CTC CAA CA-3' Antisense: 5'-TAT <i>GCT AGC</i> CCC CTT CCT TCT CCT CCT C-3'
<i>SLC19A3</i> -1957 (Full)	Sense: 5'-CGG <u>GCT ACC</u> AGA CTG TGG CTA TGA G-3'
<i>SLC19A3</i> -970	Sense: 5'-CGG <u>GGT ACC</u> TAG GGA GGC TGA GGC AGA AG-3'
<i>SLC19A3</i> -473	Sense: 5'-CGG <u>GGT ACC</u> CCA GGA AAC CAT CCC ACT CT-3'
<i>SLC19A3</i> -32	Sense: 5'-CGG <u>GGT ACC</u> TCC GGG CCA GGC AGG CTC CG-3'
<i>SLC19A3</i> +59	Antisense: 5'-CTA <i>GCT AGC</i> ATC GCT CAC TTG CCG CA-3'

Table 2
Primers for *SLC19A3* full promoter mutation constructs

Hypoxic response element locations are indicated in bold and mutations are indicated by the underline.

Primer Name	Sequence
-1336	Sense: 5'-CAA AAA AAT TAA TGT GAA <u>ATG</u> GTG GCA CCC ACC TGC AAG CC-3' Antisense: 5'- GGC TTG CAG GTG GGT GCC ACC ATT TCA CAT TAA TTT TTT TG-3'
-908	Sense: 5'-GTT CGA GTG AGC CAA GAT <u>AAT</u> GCC ATT GCA CTC CAG CCC-3' Antisense: 5'- GGG CTG GAG TGC AAT GGC ATT ATC TTG GCT CAC TGC AAC-3'
-47a	Sense: 5'-CAT ATG CAA AGC GTG GGG <u>GAA</u> TGG CCC CGG GCT C-3' Antisense: 5'-AGC CCG GGG CCA TTC CCC CAC GCT TTG CAT ATG-3'
-47b	Sense: 5'-CAT ATG CAA AGC GTG GGG GCG <u>AAG</u> CCC CGG GCT C-3' Antisense: 5'-GAG CCC GGG GCT TCG CCC CCA CGC TTT GCA TAT G-3'
-55	Sense: 5'-CAT ATG CAA AGA <u>ATG</u> GGG GCG TGG CCC CGG GCT C-3' Antisense: 5'-GAG CCC GGG GCC ACG CCC CCA TTC TTT GCA TAT G-3'
-55/47	Sense: 5'-CAT ATG CAA AGA <u>ATG</u> GGG <u>GAA</u> TGG CCC CGG GCT C-3' Antisense: 5'-GAG CCC GGG GCC ATT CCC CCA TTC TTT GCA TAT G-3'