

HYPOXIA INCREASES THE DEPENDENCE OF GLIOMA CELLS ON GLUTATHIONE

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Glutathione (GSH) is an essential antioxidant responsible for the maintenance of intracellular redox-homeostasis. As tumors outgrow their blood supply and become hypoxic, their redox-homeostasis is challenged by the production of nitric oxide (NO) and reactive oxygen species (ROS). In gliomas, the sustained import of L-cystine via the L-cystine/L-glutamate exchanger, system x_c^- , is rate-limiting for the synthesis of GSH. We show that hypoxia causes a significant increase in NO and ROS, yet without affecting glioma cell growth. This is explained by a concomitant increase in the utilization of GSH, which is accompanied by an increase in the cell surface expression of xCT, the catalytic subunit of system x_c^- and L-cystine uptake. Growth is inhibited when GSH synthesis is blocked by buthionine sulfoximine (BSO), an inhibitor of the enzyme required for GSH synthesis or when cells are deprived of L-cystine. These findings suggest that glioma cells show an increased requirement for GSH to maintain growth under hypoxic conditions. Therefore, approaches that limit GSH synthesis such as blocking system x_c^- may be considered as an adjuvant to radiation or chemotherapy.

Cellular antioxidants are important for the protection of cells against nitric oxide and reactive oxygen species (NOS/ROS) generated endogenously or through exogenously imparted stress. Glutathione (L- γ -glutamyl-L-cysteinylglycine; GSH) is one of the most abundant antioxidants in the central nervous system with concentrations in the low millimolar range (1-3). GSH is synthesized from L-cysteine, L-glutamate and glycine with L-cysteine being rate-limiting. L-cysteine is provided through the import of L-cystine (the reduced form of L-cysteine) via the Na⁺-independent L-cystine/L-glutamate exchanger, system x_c^- (4-6). System x_c^- is a

member of the family of heteromeric amino acid transporters (HATs) composed of a regulatory heavy subunit, 4F2hc/CD98, and a catalytic light subunit, xCT, which confers the specificity of this transport system. System x_c^- mediates the electroneutral uptake of L-cystine in exchange for the release of L-glutamate at a 1:1 stoichiometry. Extracellular L-cystine is rapidly reduced intracellularly to L-cysteine and incorporated into GSH, which is necessary to neutralize increased NOS/ROS as a result of changes in oxygen tension (7;8). During glioma expansion, oxygen becomes limiting due to poor tumor vasculature (9;10). In fact, tumor oxygen tension has been reported to be as low as 0.1%, resulting in tumor regions that are under chronic hypoxic conditions (11-14). System x_c^- has been shown to be up-regulated following oxidative stress (14;15). The synthesis of GSH may become critical for the protection of gliomas against oxidative damage (14-18).

In this study, we examined the role of system x_c^- mediated L-cystine uptake in providing glioma cells with sufficient L-cysteine for the synthesis of GSH. Additionally, we compared the biological importance of GSH in glioma cell growth under hypoxic (2% O₂) and normoxic (21% O₂) conditions. We show that in hypoxia, glioma cells increase NO and ROS production, which leads to a concomitant increase in L-cystine uptake via system x_c^- , as well as an enhanced cell surface expression of the xCT subunit. Sustained GSH synthesis becomes more critical for the support of glioma cell growth under hypoxic conditions compared to normoxic conditions. This is demonstrated by a ~3-fold increase in the utilization of GSH and an enhanced sensitivity of glioma cell growth to the inhibition of GSH synthesis by buthionine sulfoximine (BSO).

EXPERIMENTAL PROCEDURES

Cell Culture—D54-MG cells (WHO grade IV) were a gift from Dr. D.D. Bigner (Duke

University, Durham, NC). Routine mycoplasma tests were performed to ensure the absence of contamination. Cells were grown in Dulbecco's modified Eagle medium (DMEM/F12; Media Tech, University of Alabama at Birmingham Media Preparation Facility) and supplemented with 2 mM glutamine (Media Tech) and 7% fetal bovine serum (FBS; Hyclone, Logan, UT) at 37°C with 10% CO₂ and balanced with ambient air. For some experiments, cells were grown in DMEM 1X (Invitrogen, Carlsbad, CA—*catalog #231013-024*) supplemented with 0.5 mM sodium pyruvate, 2 mM glutamine and 7% fetal bovine serum (Media Tech, University of Alabama at Birmingham Media Preparation Facility). For hypoxic conditions, cells were grown at 37°C in a tri-gas incubator in which 2% O₂ was maintained by purging the chamber with 100% N₂ and supplementing with 10% CO₂. The pH of media was tested regularly with pH strips and a pH indicator was included in the media. The pH was maintained at 7.4 under hypoxic and normoxic conditions.

Drugs—All drugs were purchased from Sigma Aldrich unless otherwise specified (St. Louis, MO). (S)-4-Carboxyphenylglycine was purchased from Tocris Bioscience (Ellisville, MO).

Cell Proliferation—Proliferation was assessed by seeding 10,000 cells into each well of a 12-well plate (Fisher Scientific). Cells were harvested using 0.05% trypsin and re-suspended in 10 ml standard bath solution. Standard bath solution consisted of the following (in mM): 125 NaCl, 5 KCl, 1.2 MgSO₄, 1 CaCl₂, 1.6 Na₂HPO₄, 0.4 NaH₂PO₄, 10.5 Glucose and 32.5 HEPES acid. The pH was adjusted to 7.4 using NaOH and osmolarity was measured at ~300 mOsm. Three readings were made on specified days using a Coulter-Counter Cell Sizer (Beckman-Coulter, Miami, FL). Cell number was recorded per 500 µl and the mean cell number was normalized to Day 0.

Western Blot—Confluent plates of D54-MG cells were lysed using Radio-Immunoprecipitation Assay buffer (RIPA), which was supplemented with protease and phosphatase inhibitors (1:100). Protein analysis was performed using the Bio-Rad DC protein assay kit (Hercules, CA). Then, 25 µg of protein was mixed with 6X sample buffer (60% Glycerol, 300 mM Tris pH 6.8, 12 mM Ethylenediaminetetraacetic acid (EDTA), 12%

Sodium dodecyl sulfate (SDS), 864 mM 2-mercaptoethanol and 0.05% Bromophenol Blue) and boiled for 3 min. Samples were loaded into a 10% pre-cast SDS-Page gel (Bio-Rad, Hercules, CA). Gels were ran at 100 V for 90 min and transferred at 200 mA for 120 min at room temperature (RT) onto polyvinylidene fluoride paper (Millipore, Bedford, MA). Membranes were blocked in blocking buffer (5% nonfat dried milk in TBS plus 0.1% Tween 20; TBST). Blots were probed with primary antibody, goat anti-xCT overnight at 4°C (0.06 µg/ml, Abcam, Cambridge, MA). Blots were also probed with mouse anti-GAPDH (0.05 µg/ml, Abcam, Cambridge, MA). Following primary antibody incubation, blots were washed 4x for 5 min each in TBST. Next, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (2 µg/0.5ml, Santa Cruz Biotechnology Inc, Santa Cruz, CA) for 1 h at RT, followed by another wash period (4x for 5 min each in TBST) and developed using enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). Membranes were exposed using the Kodak Image Station 4000MM (Kodak, New Haven, CT).

Cytoplasmic and Nuclear Protein extraction—D54-MG cells were harvested and washed in 1X PBS. The NE-PER Nuclear and Cytoplasmic Extraction Reagents (NER and CER I, respectively) (Pierce, Rockford, IL) were used to isolate protein fractions. Protocol was followed as instructed by the manufacturer with some modification. CER I and NER were supplemented with protease and phosphatase inhibitors (1:50). Proteins were examined by Western Blot and probed with mouse anti-HIF 1-α (1.3 µg/ml, Abcam, Cambridge, MA) and mouse anti-Histone 1 (1 µg/ml, Millipore, Bedford, MA), each for 1 h at RT. To confirm proper separation between cytoplasmic and nuclear protein, blots were also probed with rabbit anti-α-tubulin (0.2 µg/ml, Abcam, Cambridge, MA).

Biotinylation—To prevent endocytosis of surface proteins, this assay was performed at 4°C. Cells were washed 2x in standard bath solution supplemented with 1 mM CaCl₂. After washing, 1.5 mg/ml Sulfo-NHS-Biotin (Pierce, Rockford, IL) was added and allowed to incubate for 30 min with occasional gentle rocking. Biotinylation was quenched with standard bath solution supplemented with 100 mM glycine and 1 mM

CaCl₂ (pH 8.0). Cells were washed once in standard bath solution and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (1:100). Protein analysis was performed using the Bio-Rad DC protein assay kit. A 2.5 mg/ml protein stock was prepared and 0.4 ml of protein was incubated with 200 µl of agarose streptavidin beads (Pierce, Rockford, IL) overnight at 4°C. The bound fraction was gently washed 5x with RIPA, re-suspended in 50 µl 6X sample buffer and boiled for 10 min to separate surface protein from beads. Samples were processed by Western Blot. Blots were probed with primary antibodies mouse anti-Na⁺/K⁺-ATPase 1 h at RT (1 µg/ml, Millipore, Bedford, MA) and goat anti-xCT overnight at 4°C.

L-cystine uptake—L-cystine uptake was performed using ¹⁴C-L-cystine as described previously with modifications (4). Uptake was performed using 2 µCi ¹⁴C- L-cystine (Perkin Elmers, Waltham, MA) with 100 µM L-cystine and was measured over 3 min. Uptake was normalized to protein, which was measured using Better Bradford Protein Assay (Thermo Fisher Scientific, Rockford, IL).

Glutathione Assay—Reduced glutathione was measured using the QuantiChrom™ Glutathione Assay Kit (DIGT-250) from BioAssay Systems (Hayward, CA). The Protocol was followed as directed by manufacturer. The QuantiChrom™ Glutathione Assay Kit measures reduced GSH. D54-MG cells grown under hypoxic or normoxic conditions were harvested and sonicated in a solution containing 50 mM NaH₂PO₄ and 1 mM EDTA. Lysates were centrifuged at 10,000 g for 15 min at 4°C and the supernatant was collected for assay. First, samples were mixed with equal volume of Reagent A, vortexed, and centrifuged for 5 min at 14,000 rpm. Next, 200 µl sample/Reagent A mixture was aliquoted into wells of a 96-well plate and 100 µl of Reagent B was added to each sample/Reagent A containing well. The Plate was incubated for 25 min at RT and read at OD_{450nm}. GSH concentration was calculated using the following formula:

$$\frac{(OD_{\text{SAMPLE}} - OD_{\text{BLANK}}) / (OD_{\text{CALIBRATOR}} - OD_{\text{BLANK}})}{100 \times n} = \text{GSH } (\mu\text{M})$$

The calibrator is equal to 100 µM glutathione and the blank is water alone. GSH was normalized to protein concentration, which was measured with the Bio-Rad DC protein assay kit.

NO/ROS detection—D54-MG cells were plated onto cover-slips and grown in hypoxia for 0, 24, 48 and 96 h. Cells were first washed 2x in HBSS containing Ca²⁺/Mg²⁺ (washing buffer). Then they were loaded with 1 µM CM-H₂DCFDA, an ROS dye (Invitrogen-C6827) or 2.5 µM DAF-FM, an NO indicator dye (Invitrogen-D23844) and 1 µM Hoechst 33342 (Invitrogen-H3570) for 15 min at 37°C. CM-H₂DCFDA detects hydrogen peroxide, superoxide anion and the hydroxyl radical. Loading buffer used was the same as washing buffer. Next, cells were washed 3x with washing buffer and allowed to recover for 10 min at 37°C. This was followed by fixation with 4% paraformaldehyde for 20 min and later 20x images were acquired using the Zeiss Axiovert 200M (München, Germany).

Data analysis—Results were graphed using Origin (v.7.5, MicroCal Software, North Hampton, MA) and analyzed using Graphpad InStat 3.00 (GraphPad Software, San Diego, CA, USA Copyright 1992-1998 GraphPad Software Inc). Significance was determined using a two-way ANOVA followed by a Tukey post hoc test. For all data sets comparing the mean of only two groups, an unpaired t-test was employed. Details of statistical analysis used in each figure can be found in their respective figure legends.

RESULTS

Glioma cells experience hypoxia at 2% O₂—To maintain redox-homeostasis, adequate synthesis of antioxidants specifically GSH, is critical for tumor cell survival (19). Previous studies show that changes in the oxygen tension within and around the tumor microenvironment leads to tumor hypoxia and modification of the redox-status by paradoxically challenging tumor cells oxidatively and/or nitrosatively (15;17;18). To mimic hypoxic conditions, cells were grown at 37°C in a tri-gas incubator with 2% O₂, 10% CO₂ and 88% N₂. For comparison, normoxic conditions were achieved in an incubator in which the temperature was set to 37°C with 10% CO₂ and balanced with ambient air. To show that D54-MG cells were indeed responding to hypoxic conditions, we examined a classical cellular response to hypoxia, namely an increase in the hypoxia inducible factor 1 alpha (HIF-1α). HIF-1α is the regulated subunit of the *HIF-1* transcription factor. Activated HIF-1α

translocates to the nucleus where it binds to its response element, and induces transcription of a number of genes involved in the cellular response to hypoxia (20). D54-MG cells were cultured under hypoxic conditions at defined time points and nuclear and cytoplasmic proteins were isolated and examined by Western Blot and probed for HIF-1 α expression (Fig. 1a). To assure efficient separation of nuclear and cytoplasmic proteins, membranes were also probed for α -tubulin. Following densitometric analysis, HIF-1 α bands were normalized to Histone 1. After 5 h of hypoxia, HIF-1 α expression increased significantly, $p < 0.001$; it remained elevated for 24 h and returned to basal levels by 48 h (Fig. 1b). This data demonstrates that 2% O₂ is sufficient to induce a hypoxic response in D54-MG cells.

Increased Utilization of GSH under hypoxic conditions—Thiol-reduced GSH acts as an electron donor to reduce oxidized proteins with the product being disulfide oxidized GSSG (21;22). As L-cysteine is rate-limiting for the synthesis of GSH, we first examined the dependency of GSH synthesis on the availability of extracellular L-cystine under hypoxic and normoxic conditions (7). D54-MG were cultured under 2% or 21% O₂ for 96 h and at 72 h, glioma cells were depleted of intracellular GSH by removing extracellular L-cystine 24 h before measuring GSH. After GSH depletion, glioma cells were treated with increasing concentrations of L-cystine for 6 h followed by GSH measurement, which was normalized to protein concentration. Results show a concentration-dependent increase in intracellular GSH with increasing concentrations of L-cystine. The half maximal concentration of L-cystine required under hypoxic and normoxic conditions was 12 μ M and 9 μ M respectively (Fig. 2a). Next, we examined the steady state GSH concentration under hypoxic and normoxic conditions. D54-MG cells were grown under hypoxic or normoxic conditions and after 72 h, culture media was changed to media without L-cystine. After 24 h, 100 μ M L-cystine was added at defined time-points and GSH was measured and normalized to protein concentrations. The results show no significant difference in steady state GSH concentration (Fig. 2b). This may be explained by an elevated rate of GSH consumption. To assess how quickly GSH is consumed, D54-MG cells were grown under hypoxic and normoxic

conditions for 96 h. Culture medium was changed to media containing 0 μ M L-cystine at 1, 3, 6, 12 and 24 h prior to determining remaining GSH concentration. This was done in order to inhibit cellular re-synthesis of GSH (Fig. 2c). This data was well fit with an exponential decay function. These fits yielded decay times of 2.87 h for hypoxic conditions as compared to 8.09 h for normoxic conditions, a significant difference, $p < 0.05$ (Fig. 2c). This data suggests that under hypoxic conditions, GSH is consumed ~ 3 times faster, possibly due to an increased requirement for the reduction of oxidized proteins and/or entry of GSH into the γ -glutamyl cycle in order to release amino acids.

Increased sensitivity to the inhibition of GSH synthesis under hypoxic conditions—The ability to maintain homeostatic balance between free radical production and detoxification by antioxidants is critical for the survival of most cell types and the loss of that imbalance can lead to cell death (23). Therefore, we examined the importance of GSH in glioma cell growth under hypoxic and normoxic conditions. To do so, cell number was measured over 4 days in the presence of either 100 μ M L-cystine, 0 μ M L-cystine, or 0 μ M L-cystine plus GSH ethyl ester (GSHee), which is a cell permeant form of reduced GSH that has been shown to increase intracellular GSH (24). Growth was determined using a Coulter-Counter Cell Sizer, which enabled us to count cell number at defined time points. In addition, a Trypan Blue exclusion assay demonstrated that cell viability was unaffected under hypoxic and normoxic conditions (data not shown). If GSH production from L-cystine is required for cell growth, supplementing the media with GSHee alone would be sufficient to maintain growth under either condition. After 4 days under hypoxic conditions, there was a 5-fold increase in cell number in the absence of L-cystine compared to a 18-fold increase under control conditions, a significant difference, $p < 0.001$. The addition of 5 mM GSHee completely rescued growth back to control conditions at days 3 and 4 (Fig. 3a). Likewise, under normoxic conditions, glioma cell number by days 2, 3 and 4 were significantly reduced in the absence of L-cystine, but were restored back to control levels by GSHee (Fig. 3b). Indeed, glioma cell growth was unaffected by hypoxic conditions, supporting the notion that for cells to maintain

normal growth rates under hypoxic and normoxic conditions, sufficient concentrations of GSH must be maintained.

To further examine the requirement of GSH for glioma cell growth, particularly under hypoxic conditions, we examined the effects of blocking GSH synthesis with BSO, an inhibitor of γ -glutamylcysteine synthetase, which is the rate-limiting enzyme in GSH synthesis. We confirmed that BSO does effectively inhibit GSH synthesis (data not shown). Under hypoxic and normoxic conditions, and in the presence of 100 μ M L-cystine, BSO inhibited glioma cell growth with an IC_{50} of 258 μ M and 119 μ M, respectively (Fig. 4a). However, in the presence of 10 μ M L-cystine, there was an overall increased sensitivity to BSO under both hypoxic and normoxic conditions when compared to cells grown in 100 μ M L-cystine. Statistical analysis revealed that BSO, in the presence of 10 μ M L-cystine has a significantly lower IC_{50} (1 μ M) under hypoxic conditions when compared to the IC_{50} (5 μ M) under normoxic conditions, $p < 0.05$ (Fig. 4b). To show that the effect of BSO is due to decreased intracellular GSH rather than other non-specific actions, we treated glioma cells with 30 μ M BSO (+/-) 3 mM GSHee. Under normoxic and hypoxic conditions and in the presence of 10 μ M L-cystine, BSO decreased cell numbers by 95% and 96%, respectively. Furthermore, exogenous application of 3 mM GSHee completely restored growth back to control levels (Fig. 4c). These results further indicate that GSH plays a critical role in glioma cell growth under hypoxic conditions and particularly at physiological concentrations of L-cystine.

Hypoxia induced Reactive Oxygen Species and Nitric Oxide Species—Hypoxia has been shown to lead to increases in free radical production, notably ROS (17;18). We examined changes in free radical production in response to hypoxic conditions as well as the effectiveness of its neutralization by GSH. To assay for ROS production, D54-MG cells were cultured under hypoxic conditions for 48 h. Glioma cells were loaded with an ROS indicator dye, CM-H₂DCFDA and Hoechst 33342 to stain nuclei. CM-H₂DCFDA is initially non-fluorescent and once it permeates live cells, it is cleaved by nonspecific intracellular esterases. In the presence of ROS, the reduced fluorescein compound is oxidized and has

excitation/emission maxima of $\sim 495/529$ nm. Representative images are shown in Fig. 5a and the analyses of cells that emit a green fluorescence are shown in Fig. 5a.1. After 48 h of hypoxia, ROS detection significantly increased from 7.7% to 41.6% (Fig. 5a.1). The specificity of CM-H₂DCFDA was assessed by treating glioma cells under hypoxic conditions with GSH, a well characterized scavenger of ROS (25;26). To determine whether GSH is able to neutralize hypoxia induced ROS, cells were cultured with 3 mM GSHee while under hypoxic conditions. GSHee significantly reduced hypoxia induced ROS by 70% (Fig. 5a.1). Similar results were observed at 96 h (data not shown)

Secondary classes of free radicals such as NOS are generated under hypoxic conditions (15). NO is capable of reacting with oxygen radicals such as O₂⁻ to form peroxynitrite (ONOO⁻) and nitrogen dioxide (NO₂) (27). NO intermediates specifically NO₂, can further react with NO to form dinitrogen trioxide (N₂O₃), a potent NOS that imparts nitrosative stress (28). NO production was assessed by loading cells with an NO indicator dye, DAF-FM diacetate, which is a cell permeant dye that once inside the cell, it is deacetylated by esterases to form DAF-FM. In the presence of NO, DAF-FM forms a fluorescent benzotriazole derivative with excitation/emission maxima of $\sim 495/515$ nm. Glioma cells grown under hypoxic conditions also demonstrated a significant increase in NO productions (Fig. 5b). After 48 h of hypoxia, NO detection significantly increased from 7.3% to 35.8% (Fig. 5b.1). Incubation with 3 mM GSHee also significantly reduced NO production after 48 h of hypoxia by 65% (Fig. 5b.1). Similar findings were observed at 96 h (data not shown). This data suggests that GSH is capable of fully neutralizing NOS/ROS generated by hypoxic conditions.

Inhibition of system x_c⁻ decreases glioma growth—Inhibition of system x_c⁻ under normoxic conditions decreases glioma cell growth and intracellular GSH (16). To further determine the significance of GSH in glioma cell growth under hypoxic conditions, we inhibited system x_c⁻ using two inhibitors, (S)-4-carboxyphenylglycine (S4CPG) and Sulfasalazine (SAS). S4CPG and SAS have been shown to effectively inhibit L-cystine uptake and decrease tumor growth (4;16;29-31). In addition, the effect of SAS on

tumor growth has been shown to be independent of NF- κ B and specifically due to L-cystine starvation (30;32). First, dose responses for both inhibitors were established in the presence of either 100 μ M or 10 μ M L-cystine. In 100 μ M L-cystine, S4CPG decreased growth under hypoxic and normoxic conditions with an IC₅₀ of 145 μ M and 126 μ M, respectively (Supp. Fig. 1a). In the presence of 10 μ M L-cystine, there was an increase in the sensitivity of glioma cells to S4CPG with an IC₅₀ of 0.80 μ M under hypoxic conditions and 2 μ M under normoxic conditions (Supp. Fig. 1b). In the presence of 100 μ M L-cystine, the IC₅₀ for SAS under hypoxic and normoxic conditions were, 440 μ M and 315 μ M, respectively (Supp. Fig. 1c). In addition, lowering extracellular L-cystine to 10 μ M increased the overall sensitivity of gliomas cells to SAS both under hypoxic and normoxic conditions with an IC₅₀ of 32 μ M and 40 μ M, respectively (Supp. Fig. 1d). These dose responses establish the efficacy of S4CPG and SAS for the inhibition of glioma cell growth both under high and low concentrations of L-cystine.

To examine whether GSHee could rescue growth inhibition by S4CPG and SAS under hypoxic and normoxic conditions, we used 1 mM GSHee and drug concentrations of S4CPG and SAS that resulted in greater than 80% growth inhibition, a growth inhibition similar to that seen the absence of L-cystine. Under hypoxic conditions and in the presence of 100 μ M L-cystine, S4CPG and SAS decreased cell number by 99% and 84% respectively (Fig. 6a & b). Although 1 mM GSHee significantly increased cell number in the presence of both S4CPG and SAS, only S4CPG restored growth completely back to control levels (Fig. 6a & b). Under normoxic conditions and in the presence of 100 μ M L-cystine, 500 μ M S4CPG and SAS decreased cell number by 98% and 95% respectively (Fig 6a & b). Furthermore, exogenous application of 1 mM GSHee was able to restore growth back to control levels in the presence of either drug.

Hypoxia increases ¹⁴C-L-cystine uptake and cell surface expression of the xCT subunit of system x_c⁻— Previous reports demonstrate that NO donors such as 3-nitroso-N-acetylpenicillamine (SNAP) and S-nitrosolglutathione (SNOG), as well as ROS donors including xanthine/xanthine oxidase and H₂O₂ increased system x_c⁻ activity.

This led us to examine how ¹⁴C- L-cystine uptake through system x_c⁻ is affected by hypoxia. After 72 h under hypoxic or normoxic conditions, glioma cells were washed in Na⁺-independent uptake solution to eliminate the contribution of Na⁺- dependent uptake systems. This was followed by the addition of Na⁺-independent uptake solution containing 2 μ Ci of ¹⁴C-L-cystine and 100 μ M L-cystine, and uptake was measured over 3 min. D54-MG cells grown under hypoxic conditions took up ~30% more L-cystine than cells grown under normoxic conditions (Fig. 7). Concentrations as low as 250 μ M S4CPG and SAS equally decreased L-cystine uptake in D54-MG (16). To investigate a possible up-regulation of any competing L-cystine transporters, we measured L-cystine uptake in the presence of a high dose of SAS to maximize inhibition of L-cystine uptake. D54-MG cells were grown under hypoxic conditions for 72 h and uptake was measured in the presence of 750 μ M SAS or vehicle. The results show that in the presence of SAS, L-cystine uptake was decreased by ~67% under hypoxic conditions and 63% under normoxic conditions (Fig. 7). This suggests that under hypoxic conditions, the majority of L-cystine transport is mediated through system x_c⁻, and the enhanced uptake may be due to an enhanced expression of system x_c⁻.

To investigate the effects of hypoxia on xCT expression, we examined total protein after glioma cells were grown under hypoxic conditions for defined periods. Cell lysates were collected, ran on a Western Blot and probed for xCT and GAPDH (Fig. 8a). Through densitometric analysis, we show that hypoxia had no effect on total protein (Fig. 8b). Next, we examined cell surface xCT expression using a biotinylation assay. D54-MG cells were grown in 2% O₂ for defined periods. At the end of the last time point, cells were brought to 4°C to stop endocytosis and surface proteins were biotin labeled and streptavidin coupled. Cells were lysed and collected for Western Blot. Blots were probed with xCT and normalized to Na⁺/K⁺-ATPase (Fig. 8c). Our results show a 3-4 fold increase in cell surface expression of the xCT subunit at 48 and 96 h, respectively (Fig. 8d). These findings suggest that following hypoxia, increased surface expression of xCT leads to enhanced L-cystine uptake.

DISCUSSION

Our results demonstrate that under hypoxic conditions, glioma cells exhibit an increased dependence on GSH for cell growth, particularly under conditions of limited L-cystine availability. GSH maintains the thiol redox potential in cells, neutralizes free radicals and serves as a reservoir for intracellular L-cysteine (33-36). We show that in the presence of 10 μ M L-cystine, BSO inhibits glioma cell growth and GSHee completely rescues growth back to control levels. This suggests that the primary role for GSH in glioma cells is redox regulation rather than protein synthesis. It is possible that under hypoxic conditions, there is an increase in oxidized proteins, possibly ribonucleotide reductase (RNR). RNR is an enzyme that catalyzes the formation of deoxyribonucleotides from ribonucleotides and is required for DNA synthesis and cell cycle progression (21;22;37). These findings are supported by data showing that under hypoxic conditions, there are indeed increases in both NO and ROS. Despite these increases in NOS/ROS, glioma cell growth was unabated provided cells were maintained under conditions that supported the *de novo* synthesis of GSH. Interestingly, some cell types are capable of synthesizing GSH from L-methionine in the absence of L-cystine by going through the transsulfuration pathway (38). In fact, some cancers are dependent on the availability of L-methionine for the synthesis of GSH exclusively, and in its absence, growth is stunted (39). However, D54-MG cells are unable to substitute L-methionine for L-cystine (data not shown), making L-cystine critical for GSH synthesis and glioma cell survival.

We hypothesize that glioma cells adjust to increased levels of NO and ROS by increasing the uptake of L-cystine in order to provide sufficient substrate for GSH synthesis. This is consistent with previous findings that demonstrate increased nitrosative and oxidative stress increases system x_c^- activity and xCT expression in retinal ganglion cells (40). Furthermore, IL-1 β potentiates hypoxic neuronal cell death via functional increase in system x_c^- activity (41). Likewise, we also found that glioma cells grown under hypoxic conditions for 72 h showed enhanced 14 C- L-cystine uptake and SAS decreased uptake by > 50%. The inhibitory effects of SAS on system x_c^- mediated

L-cystine uptake is in agreement with previous reports showing that SAS reduced system x_c^- activity in gliomas and not in astrocytes and neurons. These cell types mainly depend on L-cystine/L-cysteine transport via the Na $^+$ -dependent excitatory amino acid transporter (EAAT) systems (4;42;43).

The here reported increased system x_c^- activity under hypoxic conditions is contrary to findings in human fibroblast and in mouse peritoneal macrophages where hypoxia reduced L-cystine uptake (44;45). It is possible that fibroblast and macrophages unlike glioma cells, show a differential dependency on system x_c^- mediated L-cystine uptake in response to low oxygen. However, the increased L-cystine uptake in gliomas is readily explained by our finding that hypoxia increases cell surface expression of xCT 3-fold. Indeed, the increase at the protein level is larger than the here recorded increase in L-cystine transport, suggesting that not all xCT subunits participate in L-cystine transport. It is, for example possible that not all surface xCT associates with CD98, which is required to compose a functional transporter. From a mechanistic point of view, we suggest that glioma cells maintain a cytoplasmic reservoir of xCT, which is recruited to the plasma membrane on demand in order to meet its redox needs, i.e. GSH production. This likely represents an adaptation to the cells biological microenvironment where oxygen tension has been shown to vary considerably from 2-21% in normal tissue to as low as 0.1% within tumors (11-14).

Although, hypoxia increased L-cystine uptake, there was no increase in steady state GSH concentrations. This can be readily explained by the increased utilization of GSH observed under hypoxic conditions. GSH metabolism in the γ -glutamyl cycle and increased γ -glutamyl transpeptidase activity are possible mechanism of for GSH consumption (33;46). Interestingly, γ -glutamyl transpeptidase expression positively correlates with high-grade glioma and increased oxidative stress may increase γ -glutamyl transpeptidase expression and/or activity (47;48). Consistent with an enhanced need for GSH under hypoxic conditions, glioma cells in the presence of low L-cystine and under hypoxic conditions were also more sensitive to BSO, an inhibitor of GSH synthesis. These findings suggest that system x_c^- mediated L-cystine uptake gains even greater

importance in the growth control of gliomas as they outgrow their blood supply and the tumor experiences hypoxia. Although, hypoxia was the only exogenously imparted stress in this study, one can extrapolate from our findings that under hypoxic conditions, the ability of glioma cells to maintain homeostatic GSH levels may render them more resistant to radiation and chemotherapeutic approaches. Of note, radiation damage is mainly due to the generation of hydroxyl radicals which are effectively neutralized by GSH, and many gliomas are indeed highly resistant to radiation therapy (49;50). Similarly, resistance to chemotherapeutic drugs is common in gliomas and is thought to be due to the activity of the multidrug resistant gene that encodes for a transporter that require conjugation of the compound to GSH in

order to be transported (51). Hence, GSH production under hypoxia is likely to enhance both radiation and chemoresistance of gliomas. It raises the question of whether an increase in free radical production in response to radiation therapy and chemotherapeutic drugs is signaling glioma cells to increase system x_c^- in order to combat its new redox-status. Previous findings suggests that SAS may be an excellent drug candidate to target system x_c^- in gliomas (16). The findings here suggest that the target is indeed up regulated under hypoxic conditions. Taken together, a strong argument can be made that the inhibition of system x_c^- via SAS or similar drugs should be considered as adjuvant treatment for patients undergoing radiation and/or chemotherapy to enhance treatment effectiveness.

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FOOTNOTES

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The abbreviations used are: GSH, glutathione; SAS, sulfasalazine; S4CPG, (S)-4-carboxyphenylglycine; NOS, nitrogen oxide species; ROS, reactive oxygen species; BSO, buthionine sulfoximine; NER, Nuclear Extraction Reagents; CER, Cytoplasmic Extraction Reagents; RIPA, Radio Immunoprecipitation Assay; NO, nitric oxide; RT, room temperature; EDTA, Ethylenediaminetetraacetic acid; SDS, Sodium dodecyl sulfate.

FIGURE LEGENDS

FIGURE 1. Increased nuclear HIF-1 α expression in response to 2% O₂. *A*, Representative blot of nuclear expression of HIF-1 α after treatment with 2% O₂. *B*, Densitometric analysis of four independent experiments. Two-way ANOVA followed by Tukey post hoc analysis was used to determine significance, ***p*<0.01, ****p*<0.001 and N=4.

FIGURE 2. Hypoxia increases the utilization of GSH. *A*, L-cystine dependent GSH synthesis under normoxic (21% O₂) or hypoxic (2% O₂) conditions. *B*, GSH concentration as a function of time after the addition of 100 μ M L-cystine. *C*, Rate of GSH utilization as a function of time after the exclusion of L-cystine. Two-sample t-test was used to analyze the difference between IC₅₀ for GSH and decay constant, N=4.

FIGURE 3: Glioma cell growth is dependent on GSH. *A & B*, Glioma cell growth under normoxic (21% O₂) or hypoxic (2% O₂) conditions is dependent on GSH in the absence of L-cystine. Two-way ANOVA followed by Tukey post hoc analysis was used to determine significance, **p*<0.05, ***p*<0.01, ****p*<0.001, N=4.

FIGURE 4: Increased sensitivity of glioma cell growth to the inhibition of GSH. *A & B*, Inhibition of GSH synthesis by BSO in 100 μ M L-cystine under normoxic (21% O₂) or hypoxic (2% O₂) conditions, N=5. *B*, Increased sensitivity to the inhibition of GSH synthesis by BSO in 10 μ M L-cystine under normoxic or hypoxic conditions, N=6. *C*, Treatment of D54-MG cells with 3 mM GSHee restores growth inhibition by BSO under normoxic or hypoxic conditions, N=3. Two-sample t-test was used to analyze the difference between IC₅₀ for BSO.

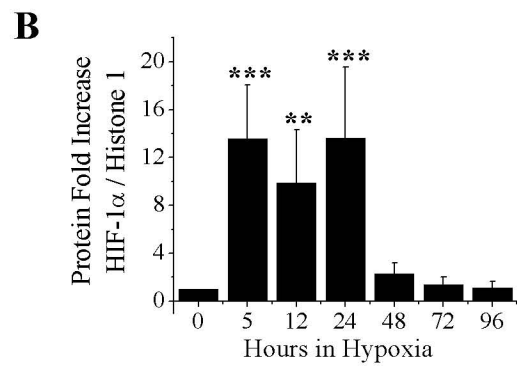
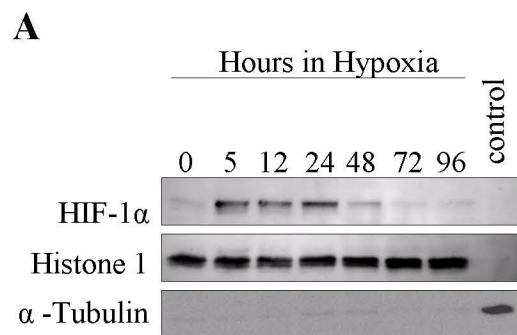
FIGURE 5. Hypoxia induced ROS and NO. *A & B*, Representative images of ROS and NO positive D54-MG cells as detected by indicator dyes, CM-H₂DCFDA and DAF-FM, respectively. Glioma cells were grown under normoxic (21% O₂) or hypoxic (2% O₂) conditions for 0 and 48 h (+/-) 3 mM GSHee. *A.I & B.I*, Analysis of ROS and NO positive D54-MG cells, respectively. Two-way ANOVA followed by Tukey post hoc was used to determine significance, ****p*<0.001, ***p*<0.01, N=3, scale bar is 50 μ m.

FIGURE 6. GSH restores growth inhibition by SAS and S4CPG. *A & B*, Inhibition of system x_c⁻ with 500 μ M S4CPG or SAS under normoxic (21% O₂) or hypoxic (2% O₂) conditions. 1 mM GSHee restored growth inhibition of S4CPG and SAS back to control. Two-ANOVA followed by Tukey post hoc was used to determine significance, ****p*<0.001, ***p*<0.01, **p*<0.05, N=3.

FIGURE 7. Hypoxia enhances ¹⁴C-L-cystine uptake via system x_c⁻. *A*, Uptake of ¹⁴C-L-cystine in D54-MG cells cultured either under normoxic (21% O₂) or hypoxic (2% O₂) conditions for 72 h (+/-) 750 μ M SAS. Two-way ANOVA followed by Tukey post hoc was used to determine significance, ****p*<0.001, **p*<0.05, N=8.

FIGURE 8. Hypoxia increases surface expression of xCT in D54-MG cells. *A & C*, Representative blots of D54-MG cells grown under hypoxic (2% O₂) conditions and probed for xCT (*A*-total & *C*-surface protein). *B & D*, Densitometric analysis of four independent experiments (*B*-total & *D*-surface) Two-way ANOVA followed by Tukey post hoc was used to determine significance, **p*<0.05, N=4.

Figure 1



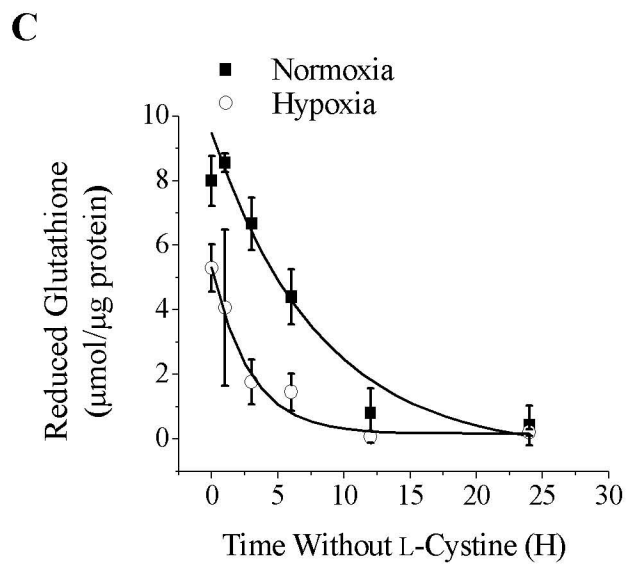
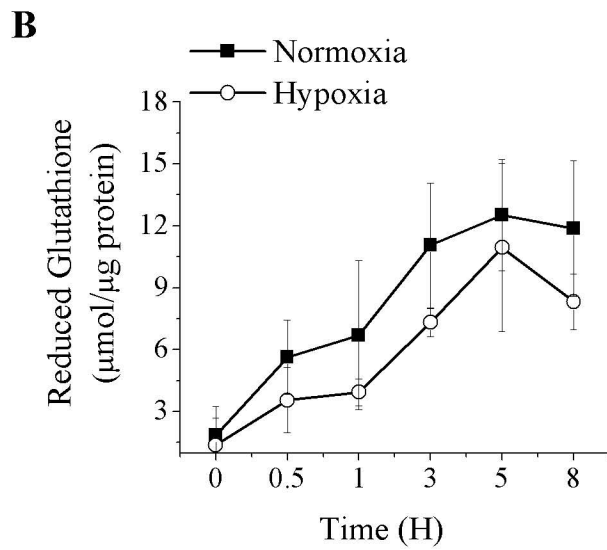
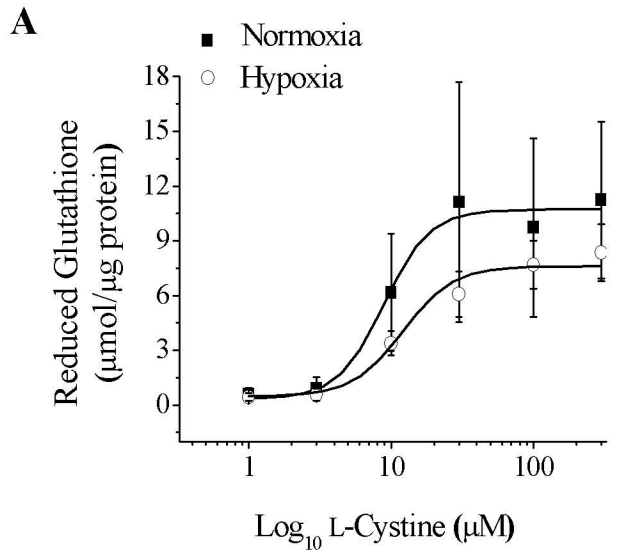


Figure 2

Figure 3

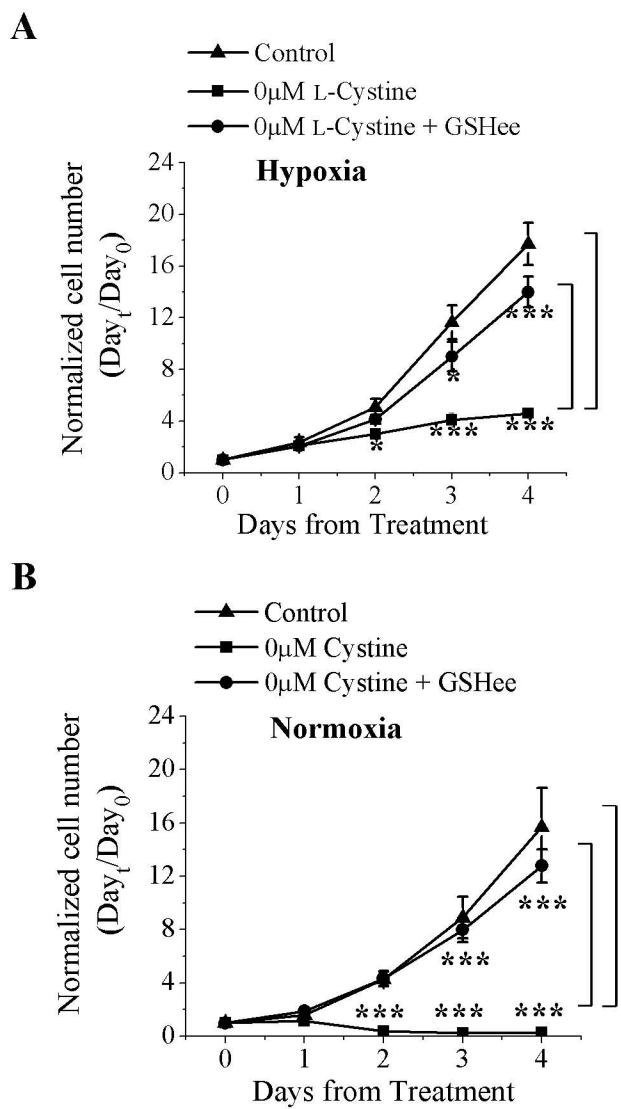


Figure 4

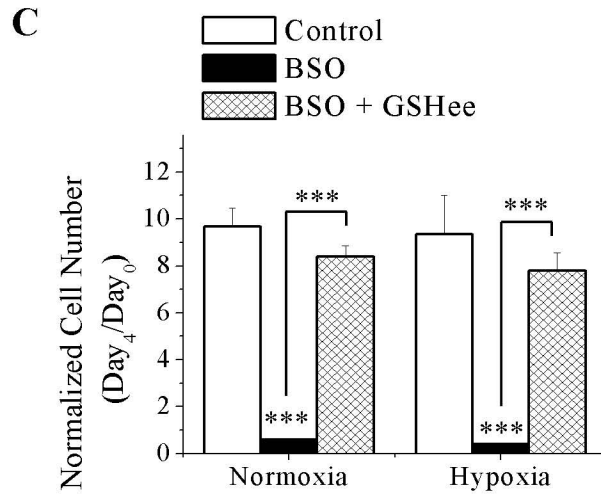
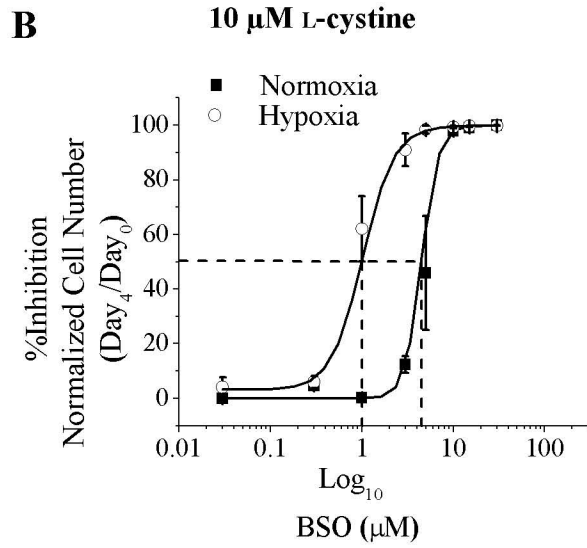
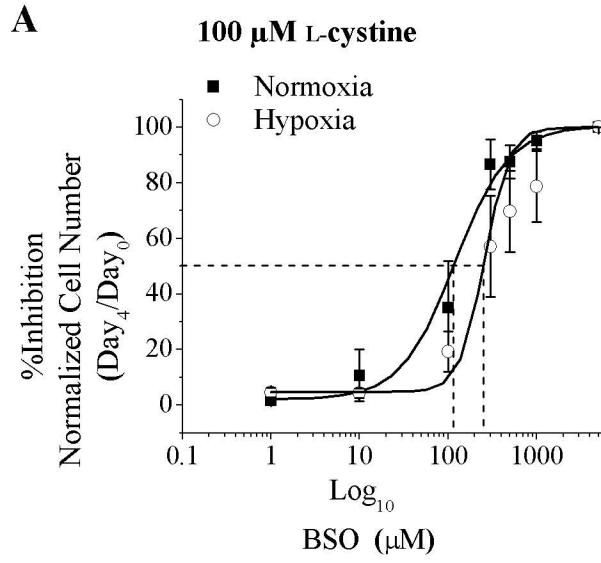
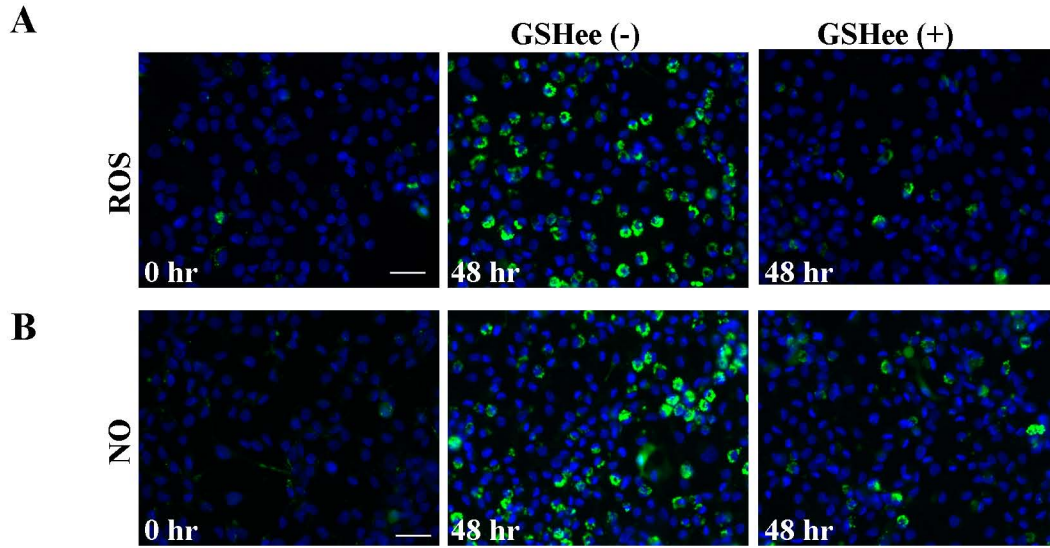
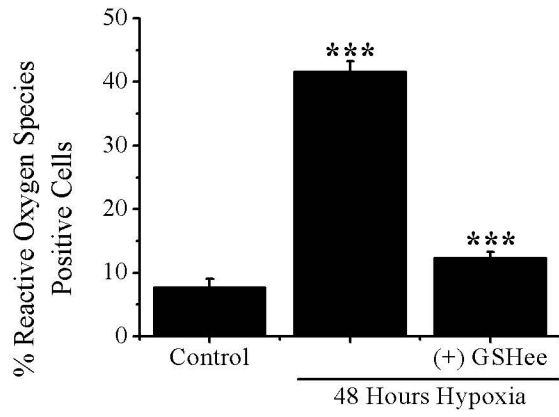


Figure 5



A.1



B.1

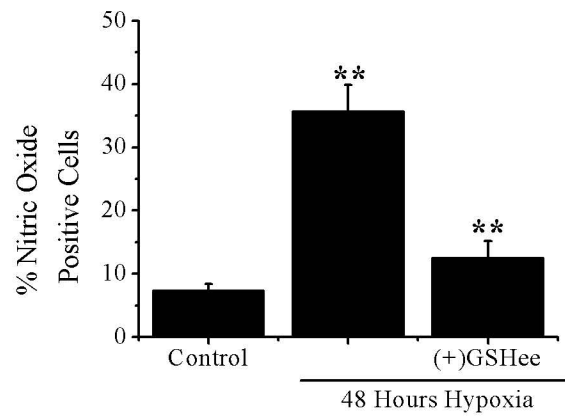
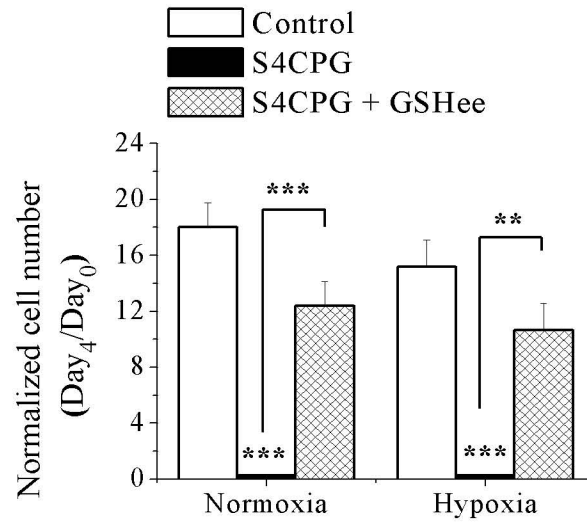


Figure 6

A



B

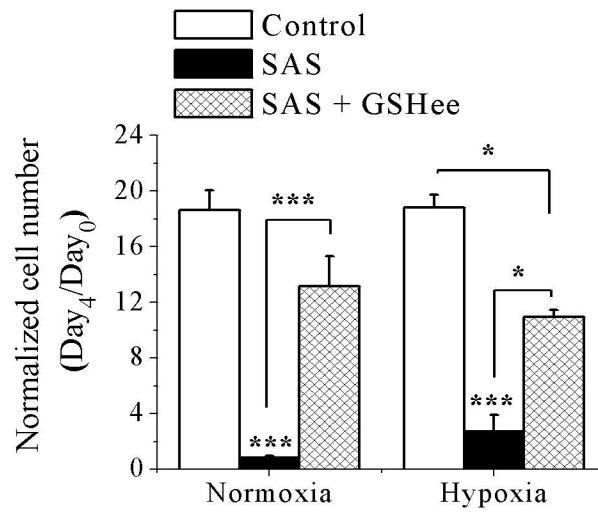


Figure 7

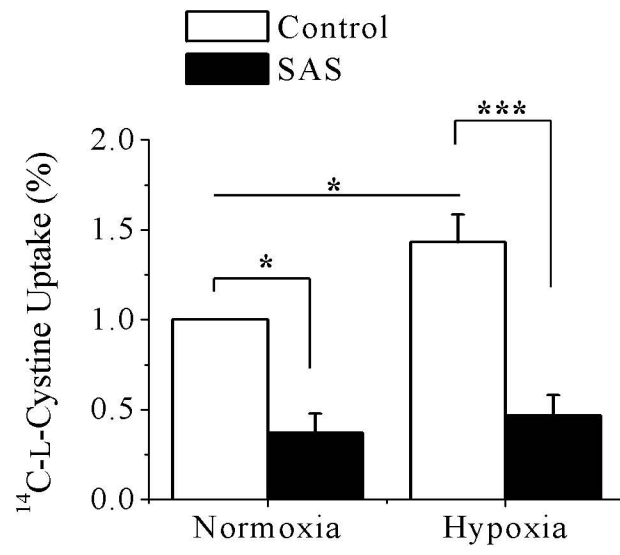


Figure 8

