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Hydrogen Peroxide Triggers an Increase in Cell Surface Expression of System x_c^- in Cultured Human Glioma Cells

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Abstract

System x_c^- exchanges extracellular cystine for intracellular glutamate across the plasma membrane of many cell types. One of the physiological roles of System x_c^- is to provide cystine for synthesis of the antioxidant glutathione. Here we report that hydrogen peroxide (H_2O_2) triggers the translocation of System x_c^- to the plasma membrane within 10 min of the initial exposure. Specifically, we observed a three-fold increase in ^{35}S -L-cystine uptake following a 10 min exposure to 0.3 mM H_2O_2 . This effect was dose-dependent with an EC_{50} for H_2O_2 of 65 μM . We then used cell surface biotinylation analysis to test the hypothesis that the increase in activity is due to an increased number of transporters on the plasma membrane. We demonstrated that the amount of transporter protein, xCT, localized to the plasma membrane doubles within 10 min of H_2O_2 exposure as a result of an increase in its delivery rate and a reduction in its internalization rate. In addition, we demonstrated that H_2O_2 triggered a rapid decrease in total cellular glutathione which recovered within two hours of the oxidative insult. The kinetics of glutathione recovery matched the time course for the recovery of xCT cell surface expression and System x_c^- activity following removal of the oxidative insult. Collectively, these results suggest that oxidants acutely modulate the activity of System x_c^- by increasing its cell surface expression, and that this process may serve as an important mechanism to increase *de novo* glutathione synthesis during periods of oxidative stress.

Key Words: Cystine/glutamate exchanger, neurotransmitter transporter, membrane trafficking, oxidative stress, glutathione, ischemia

Abbreviations:

AMPA α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BSA bovine serum albumin

CPG *S*-4-carboxyphenylglycine

DAT Dopamine transporter

DCFH-DA 2',7'-dichlorodihydrofluorescein diacetate

DEM diethylmaleate

DS donkey serum

EEA1 early endosome antigen

EAAC1 excitatory amino acid carrier 1

EAAT excitatory amino acid transporters

ENaC epithelial sodium channel

EV empty vector

FBS fetal bovine serum

GABA γ -aminobutyric acid

GAT1 γ -aminobutyric acid transporter 1

HAAT heterodimeric amino acid transport

H₂O₂ Hydrogen peroxide

Nrf2 Nuclear factor erythroid 2-related factor 2

PBS Phosphate buffered saline

RIPA Radioimmunoassay

SDS Sodium dodecyl sulfate

TfR Transferrin receptor

1. Introduction

Cells utilize several antioxidant defense systems to counteract the reactive oxygen intermediates that are by-products of normal cellular metabolism (see Sies, 1997 for review). Without these systems, the reactive oxygen species would rapidly damage cellular lipids, proteins, and DNA, leading to oxidative stress and eventually cell death. Glutathione is a tripeptide composed of glutamate, cysteine and glycine and is one of the most important small molecule antioxidants. Glutathione is typically maintained in millimolar concentrations with cells. As such, depletion of cellular glutathione levels rapidly leads to oxidative stress, and if severe, oxidative cell death (Sies, 1997). Thus, it is not surprising that disorders in glutathione metabolism are associated with many neurodegenerative diseases (see Aoyama and Nakaki, 2013 for review).

System x_c^- is a membrane transport system that plays a significant role in maintenance of cellular glutathione levels and protection from oxidative stress (Kato et al., 1993, 1992a; McBean, 2002; Sato et al., 2000). Specifically, System x_c^- is a Na^+ -independent, Cl^- -dependent transporter which exchanges extracellular cystine for intracellular glutamate (Bannai and Kitamura, 1980; Patel et al., 2004) in the meninges, and astrocytes in many regions of the brain (including the cortex, hippocampus, cerebellum, and the striatum). It is also active in the duodenum and kidney (Burdo et al., 2006; Chase et al., 2001; Pow, 2001; Shih and Murphy, 2001). In these tissues, cystine, which is internalized by System x_c^- , is rapidly reduced to cysteine, the rate limiting reagent for the synthesis of glutathione (Lu; McBean, 2012). As such, pharmacological inhibition of System x_c^- in cultured cells results in decreased cellular glutathione levels, the development of oxidative stress and cell death (Albrecht et al., 2010; Kato et al., 1992a; Murphy et al., 1990; Pereira and Oliveira, 1997). Furthermore, decreases in System x_c^- expression are associated with loss of glutathione and an increase in oxidative stress (Carpi-Santos et al., 2015; Nabeyama et al., 2010; Sato et al., 2005; Shih et al., 2006a), and induction of System x_c^- in cell cultures leads to increases in intracellular glutathione levels and greater protection from oxidative stress (Miyazaki et al., 2016; Shih et al., 2006b). In turn, the redox environment of the cell appears to regulate the basal level of System x_c^- expression. For example, chronic exposure of cultured cells to oxidative agents or glutathione synthesis inhibitors leads to increased System x_c^- expression over the course of 24 hours (Bannai, 1984; Miura et al., 1992; Ruiz et al., 2003; Sato et al., 2001). This process has been shown to be a result of Nrf2-mediated transcriptional activation of xCT, one of two subunits that comprise System x_c^- (Bannai and Ishii, 1982; Ishii et al., 2000; Sasaki et al., 2002; Shih et al., 2003).

Despite the fact that System x_c^- plays such a pivotal role in regulating glutathione synthesis, the short-term regulation of System x_c^- by oxidants is not understood. However, System x_c^- activity has been shown to increase within minutes of onset of ischemia in synaptic membrane fractions (Koyama et al., 1995), mixed cortical cell cultures (Fogal et al., 2007), cortical brain slices and *in vivo* in the cortex and

striatum (Soria et al., 2014). These data suggest that oxidants may acutely regulate System x_c^- activity, thereby providing a rapid mechanism by which cells may increase cystine import and glutathione synthesis prior to Nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated transcriptional upregulation of System x_c^- expression. In the present study, we sought to characterize the acute regulation of endogenously expressed System x_c^- by hydrogen peroxide (H_2O_2) in cultured human astrocytoma cells (U-138MG). We chose to study cultured astrocytes specifically given that 1) System x_c^- is primarily expressed in astrocytes in the brain and 2) astrocytes play a central role in controlling neuronal glutathione levels. Thus, astrocytes represent an excellent system for addressing questions related to the acute regulation of System x_c^- by oxidants. Our results indicate that H_2O_2 triggers the rapid trafficking of endogenously expressed System x_c^- to the cell surface, resulting in a three-fold increase in cystine uptake within 10 min of H_2O_2 exposure. Moreover, we demonstrated that the kinetics of increased cystine transport and cell surface expression match the kinetics of the recovery of intracellular glutathione following acute hydrogen peroxide exposure. Therefore, these findings suggest that the acute regulation of System x_c^- trafficking may serve as an important component of the defense arsenal utilized by glial cells to maintain cellular glutathione levels following an oxidative insult. A portion of this work has previously been presented in poster form (Goltz et al., 2008; Sheldon et al., 2006).

2. Experimental Procedures

2.1 Cell Culture. Human glioma U-138MG cells were maintained in Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% non-essential amino acids solution, and 1% penicillin/streptomycin in 100-mm dishes. COS-7 cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, 1500 mg/L $NaCO_3$, 10% FBS and 1% penicillin/streptomycin in 100-mm dishes. Medium was changed every two or three days, and cultures were split 1:6 every 6-8 days. U-138MG cells and COS-7 cells were obtained from the American Type Culture Collection. Tissue culture media and chemicals were purchased from Life Technologies.

2.2 Human myc-xCT and HA-CD98 constructs: Human xCT was cloned into pCR2.1 using the TA Cloning Kit (Invitrogen) and sub-cloned into pCMV-3Tag-2A (Clontech) vector at the EcoR1/HindIII site by adding EcoR1 and HindIII restriction sites to the full length xCT cDNA using appropriate primers with PCR. Human CD98 was initially cloned into pCR2.1 using the TA Cloning Kit (Invitrogen) and later subcloned into pCMV-HA-N (Clontech) at the EcoR1/NotI site by adding EcoR1 and NotI restriction sites to the full length cDNA using appropriate primers with PCR. Insertion of human xCT and human CD98 was confirmed using restriction digest analysis followed by complete DNA sequencing of the inserts and vector cloning site.

2.3 Heterologous expression of human myc-xCT and HA-4F2HC in COS-7 cells: Transfection-quality cDNA was produced using the Pure Yield Plasmid Midi-prep system (Promega). myc-xCT and HA-4F2HC were co-transfected into COS-7 cells using Lipofectamine 2000 (Life Technologies). Six hours later the media was replaced with antibiotic-free and FBS-free media which was maintained for 24-48 hours prior to the initiation of cell surface biotinylation assays.

2.4 Development of anti-xCT antibody. System x_c^- belongs to a family of heterodimeric amino acid transport (HAAT) proteins (SLC7a11) that includes nine different members (for review see Chillarón et al., 2001; Palacín and Kanai, 2004). Like all members of the HAAT family, System x_c^- requires the expression of two proteins, a 50 kD light chain protein (xCT) that is linked through a disulfide bond to a 98 kD type II glycosylated heavy chain protein, 4F2HC (also named CD98, FRP-1, and SLC3A2) (Sato et al., 2000, 1999). Polyclonal anti-peptide antibodies were generated against the protein-conjugated synthetic peptide (KPVVSTISKGGYLQG) in rabbit corresponding to amino acids 3-18 in the N-terminal region of xCT, and the antibodies were affinity purified from the crude antiserum using methods previously described (Rothstein et al., 1994; Furuta et al., 1997).

The specificity of the newly developed anti-xCT antibody was initially tested on cell lysates prepared from the cultured human glioma cell line, U-138MG, which exhibits a high level of endogenous System x_c^- activity (Ye et al., 1999). Western blot analysis of U-138MG cell lysates with anti-xCT antibody yielded a prominent band at 40 kD band, similar to the immunoreactivity observed by Sieb, et al. (Seib et al., 2011) (Figure 1A). We examined the specificity of the antibody by two methods. Since xCT protein expression and System x_c^- activity have been reported to be upregulated by exposure of cultured cells to oxidizing agents (Bannai, 1984; O'Connor et al., 1995; Sato et al., 1998), we examined xCT expression in U-138 MG cells treated with 0.1 mM diethylmaleate (DEM) for 24 hours. ^{35}S -L-cystine transport doubled following DEM treatment, increasing from 58 ± 9 pmols/mg protein/min under baseline conditions to 128 ± 7 pmols/mg protein/min). We also observed a doubling of the immunoreactivity of the 40 kD band as expected following DEM treatment and the appearance of less intense bands at 27 kD, 95 kD (potential xCT multimer) and 135 kD (xCT:CD98 dimer), similar to those detected with other xCT antibodies (Burdo et al., 2006) (Figure 1a). In addition, we also overexpressed 3Xmyc-xCT in COS-7 cells and performed a biotinylation assay to separate membrane proteins from intracellular proteins. We then probed for xCT with the anti-xCT antibody. We only observed one band near 43 kD band in myc-xCT transfected cells, but not in empty vector (EV) transfected cells when probed with the anti-xCT antibody (Figure 1B). Given that the molecular weight of the myc tag is 1.2 kD, the addition of a 3X myc-tag to xCT would be expected to produce a 43.6 kD band. To confirm that this band corresponded to myc-xCT and to ensure that the protein was expressed both intracellularly and on the membrane as one would expect for System x_c^- , we transfected COS-7 cells with either myc-xCT or EV and performed a cell

surface biotinylation assay prior to Western blot analysis. When probed with anti-myc antibody, again only one specific band at 43 kD was also observed in both the intracellular and membrane fractions from myc-xCT transfect cells, while only the appearance of a non-specific (NS) band was observed in the intracellular fraction of the EV-transfected cells (Figure 1C). (We subsequently used this NS as a protein loading control and to confirm separation of membrane and intracellular fractions.) Finally, the newly developed xCT antibody (1:50) was also tested for its use in immunocytochemistry applications in COS-7 cells transfected with the myc-xCT construct (Figure 1D). After fixation, permeabilization, and blocking in PBS with 5% Donkey Sera (PBS/DS), COS-7 cells were treated with primary antibodies rabbit anti-xCT (1:50) and mouse anti-myc (1:100; Santa Cruz Biotechnology 9E10) overnight at 4°C. Cells were washed 3 times with PBS/DS and then incubated with FITC-conjugated donkey anti-rabbit IgG and Alexa-Fluor 542 conjugated donkey anti-goat IgG (1:100) for 2 hours (Jackson ImmunoResearch). We observed that xCT expression is punctate and appears to be distributed between the plasma membrane and intracellular compartments as we demonstrated in our cell surface biotinylation analysis (Figure 1C). In addition, we only observe green immunostaining in myc-xCT transfected COS-7 cells which co-localizes with red immunostaining for the myc epitope (Figure 1D). Given that the primary antibodies for myc and xCT were generated in different species and that the Pearson Correlation coefficient indicates strong colocalization between myc and xCT immunostaining (0.979), we are confident that the newly developed anti-xCT antibody specifically reacts with xCT and does not exhibit any non-specific binding.

2.5 Biotinylation of cell surface proteins. Biotinylation of cell surface proteins was performed using a method that was adapted from Fournier, et al. (Fournier et al., 2004) and Loder, et al. (Loder and Melikian, 2003). U-138MG cells or COS-7 cells were grown to 90% confluence in 35 mM dishes and were serum-starved for 24 hours prior to the experiment to synchronize them in the G₀/G₁ phase of the cell cycle. Serum-starved cells were treated with the indicated dose of H₂O₂ (Sigma) or pre-warmed culture medium (vehicle) and placed back in the incubator for 10 min. Plates were then removed, placed on ice and rinsed twice with ice-cold phosphate buffered saline (PBS) with 1 mM magnesium and 0.1 mM calcium (PBS-Ca/Mg). The plates were gently rotated at 4°C for 60 minutes in 0.5 ml of membrane-impermeant biotinylation solution (EZ link Sulfo-NHS-Biotin, 1 mg/ml in PBS-Ca/Mg, Pierce, Rockford, IL). The biotinylation solution was aspirated, the plates were rinsed twice with ice-cold PBS-Ca/Mg with 100 mM glycine (Sigma, St. Louis, MO), and incubated with 1 ml of PBS-Ca/Mg with glycine for 25 minutes at 4°C with gentle shaking. Next, the plates were rinsed twice with ice-cold PBS-Ca/Mg, and 0.3 ml of radioimmunoprecipitation assay (RIPA)/lysis buffer with protease inhibitors (150 mM NaCl, 1 mM EDTA, 100 mM Tris-HCl, pH 7.4, 1% Triton-X, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 µg/ml leupeptin, 250 µM PMSF, 1 µg/ml aprotinin, 1 mM iodoacetamide; all chemicals from Sigma, St. Louis, MO) was added to the plates and rotated for 1 hour at 4°C to lyse the cells.

The lysates were transferred to microcentrifuge tubes and centrifuged at 12,400 rpm for 20 minutes at 4°C. Aliquots of the lysate were taken for protein concentration analysis (BCA Protein Assay Kit, Pierce) and for Western blot analysis (“lysate” fraction). Aliquots of lysate were added to equal volumes of avidin bead suspension (100 µl of lysate in 100 µl of Ultralink Immobilized Monomeric Avidin, Pierce). The avidin-lysate solution was rocked overnight at 4°C, and subsequently centrifuged at 12,400 rpm for 15 minutes. A sample of the supernatant was taken for Western blot analysis (“intracellular” fraction). The avidin beads were resuspended in RIPA/lysis buffer with protease inhibitors and centrifuged at 12,400 rpm for 5 minutes. The supernatant was discarded, and the beads were washed twice in 0.3 ml of high salt-wash buffer (0.1% Triton X-100, 500 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5) and once in 0.3 ml of no salt-wash buffer (50 mM Tris, pH 7.5). The beads were incubated in 2X sample buffer for 10 minutes at room temperature and 30 minutes at 37°C, and subsequently centrifuged at 12,400 rpm for 5 minutes. A sample of the supernatant was saved for Western blot analysis (“membrane” fraction). All of the samples taken for Western blot analysis were stored in equal volumes of sample buffer (4% SDS, 120 mM Tris, 0.02% bromophenol blue, 20% glycerol, 2% β-mercaptoethanol, pH 6.8) and frozen at -20°C.

2.6 Delivery of xCT to the plasma membrane. Biotinylation of cell surface proteins was completed under trafficking-permissive conditions using an adaptation of the methods used to measure the constitutive delivery rate the Na⁺-dependent glutamate transporter, EEAC1 (Fournier et al., 2004) and the γ-aminobutyric acid (GABA) transporter, GAT1 (Wang and Quick, 2005). Cultured U-138MG cells grown to 90% confluence in 35 mM plates and serum-starved for 24 hours. Cells were washed two times in PBS-Ca/Mg pre-warmed to 37°C and treated with 0.5 mL of pre-warmed biotinylation solution at 37°C for the indicated period of time. In some experiments, 0.3 mM H₂O₂ in PBS was added for 10 min and washed prior to adding the biotinylation solution for the indicated time. The biotinylation solution was then aspirated and cells were rinsed two times in ice-cold PBS-Ca/Mg containing glycine to terminate protein trafficking. Biotinylated proteins were subsequently separated from intracellular proteins as describe above.

2.7 Western blot. Each fraction (lysate, intracellular and membrane) from the biotinylation experiments was electrophoresed on a 4-12% SDS-polyacrylamide gel (Invitrogen) and transferred to a nitrocellulose membrane (GE Amersham) at 25 volts for 2 hours. The membranes were blocked with 5% bovine serum albumin (BSA) protein in TBS with 0.05% Tween -20 (TBS-T) for 1 hour. The membranes were probed with rabbit anti-xCT (1:400), mouse anti-CD98 (1:200) (Abcam), or rabbit anti-actin (1:5,000) (Sigma) in 5% BSA in TBS-T (0.1%) in overnight. The membranes were washed in TBS-T (0.05%) 4 times for 10 minutes each time and incubated with the appropriate horseradish peroxidase-conjugated goat secondary IgG diluted 1:50,000 in 5% BSA in TBS-T (0.1%). The membranes were washed 4 times for 10 minutes each time in TBS-T (0.05%) and visualized using Lumigen TMA-6

(Lumigen) and Kodak BioMax Light Film or digitally using a UVP Bio-Chemi Gel Documentation System.

2.8 ELISA Assay for Internalization of xCT from the plasma membrane. The internalization kinetics of xCT were measured using an adaptation of an ELISA-based assay previously described to study internalization rates of many different types of receptors, including α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Lin et al., 2000; H. Y. Man et al., 2000), α -adrenergic receptors (Wang et al., 2007) and epidermal growth factor receptors (Johns et al., 2002). U-138MG cells were grown to 90% confluence in 12-well dishes, serum-starved for 24 hours, and washed two times with ice-cold PBS-Ca/Mg to halt protein trafficking. Cell surface xCT proteins were labeled by treating cells with PBS-Ca/Mg containing an anti-xCT antibody directed to an extracellular region of the protein (SCBT, Q-18, 10 μ g/mL, raised in goat) at 4°C for 1 hour. The cells were then washed two times in ice-cold PBS Ca/Mg, warmed to 37°C (trafficking permissive conditions) and treated with either saline or 0.3 mM H₂O₂ for the indicated period of time. The cells were fixed for 5 min with 2% paraformaldehyde in PBS, washed two times with PBS, and treated with a horseradish peroxidase-conjugated donkey-anti goat antibody (Jackson Immuno, 1:1000) for 1 hour. Finally, the cells were washed two more times with PBS and treated with chromogenic substrate (OPD, Sigma), for 2 min and the reaction was quenched with 0.2 volumes of 3 N HCl. The absorbance at 492 nm of each sample was then measured. The relative internalization was calculated by dividing the absorbance of each sample by the average absorbance measured in samples which were assayed immediately following the second wash step to remove the primary antibody, i.e. samples in which the temperature was not raised to trafficking-permissive conditions following primary antibody treatment (n=6).

2.9 Measurement of Intracellular H₂O₂. U-138MG cells were grown to 95% confluence in 96-well plates and serum starved for 24 hours prior to the initiation of the assay. Cells were treated with 100 μ M cell permeable 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma) for 1 hour in serum-free culture medium. The cells were washed with PBS and treated with 0.3 mM H₂O₂ or an equal volume of PBS for 10 min and then washed with PBS. The fluorescence emission (529 nm; excitation 495 nm) in each well was monitored every 2 minutes for a total of 2 hours before, during and following H₂O₂ or PBS treatment. The fraction initial emission was calculated for each well at each time point (fraction baseline DCF signal = emission at time x – emission at time 0) / emission at time 0).

2.10 Immunocytochemistry. U-138MG cells were grown to 95% confluence on cover slips and were fixed with 3% paraformaldehyde in PBS containing 5% donkey sera (PBS/DS) for 15 min and permeabilized for 5 min with 0.1% Triton-X in PBS/DS. Cells were incubated with primary antibody for anti-xCT (1:50) and/or anti-early endosome antigen (EEA1) (1:100; mouse polyclonal, BD Biosciences) or anti-CD98 (goat polyclonal from Abcam, 1:100) overnight at 4°C. Cells were washed 3 times with

PBS/DS and then incubated with FITC-conjugated donkey anti-rabbit IgG and/or Alexa-Fluor 542 conjugated donkey anti-goat IgG (1:100) for 2 hours (Jackson ImmunoResearch) at 4°C. The cells were washed four times with PBS for 10 min and the coverslips were mounted on slides with ProLongGold (Life Technologies) and prepared for viewing. Slides were viewed using a Zeiss Axiovert 200M fluorescence microscope with an ECPlanNeoFlura 100X objective (1.3 N.A.) with ApoTome for deconvolution. Images were captured using a AxioCam MRM camera and Axiovision software. Pearson coefficient measurements and were performed using NIS Elements Imaging Software Version 4.13. For whole cell co-localization measurements, the entire cell was identified as a region of interest (ROI). For examining the membrane co-localization, ROI were measured along 20-pixel width band along the periphery of the cell.

2.11 Uptake Assays U-138MG cells or COS-7 cells were plated in 12-well dishes and allowed to grow to 90-95% confluence. Cells were serum-starved for 24 hours prior to the assay. Cells were treated with fresh, serum-free culture medium containing the indicated concentration of H₂O₂ for 10 minutes prior to the uptake assay. The control cells were treated with fresh, serum-free culture medium without H₂O₂. The cells were then washed three times with PBS at 37°C. Cells were treated with the indicated concentration of L-cystine containing ³⁵S-L-cystine (0.5 µCi/mL) (GE Amersham) for 2 minutes in the presence and absence of the competitive System x_c⁻ non-transportable inhibitor, S-4-carboxyphenylglycine (CPG) (Tocris). The cells were washed three times with ice-cold PBS and then solubilized with 0.1 M sodium hydroxide. A portion of the sample was combined with CytoScint (Fisher Scientific) and analyzed using a scintillation counter. A second portion of the sample was used to measure the protein content of the sample using the BCA Protein Assay Kit (Pierce, Grand Island, NY). Each sample was then normalized to total protein content.

2.12 Glutathione Analysis Cultured U183MG cells were grown to 95% confluence on 20 mm culture plates. At time zero, the cell culture medium was removed and cells were treated with warmed culture medium containing 0.3 mM H₂O₂ for 10 min or warmed cell culture medium lacking H₂O₂ (control). After the 10 minute incubation, the culture medium from treatment and control plates was removed and fresh, warmed culture medium was added back to the plates. The plates were placed back in the incubator, and glutathione levels in the cells were then measured as described below at the indicated times.

The cells were washed with 1 mL of PBS, and 200 µL of 10 mM HCl was added and the plates were scraped. The cell suspension was transferred to a microcentrifuge tube and subjected to three cycles of freeze/thaw in liquid nitrogen. A 50 µL aliquot was removed for measurement of protein content. To the remaining sample, 5% of metaphosphoric acid (Sigma) was added to deproteinate the sample. The sample was centrifuged and triethanolamine (Sigma) was added to the supernatant to neutralize the

sample. Total cellular glutathione was measured using an adaptation of the procedure developed by Tietze (Tietze, 1969). Each sample was then normalized to total protein content.

2.13 Trypan Blue Cell Survival Assay Cultured U183MG cells were grown to 95% confluence in 12-well plates. Cells were removed from the plate using 0.025% trypsin/ 0.01% EDTA, pelleted and re-suspended in 100 μ L of PBS. The cell suspension was combined in a 1:1 ratio with 0.4% Trypan blue (Sigma, mixed and then added to a hemocytometer. Blue cells (dead) and clear cells (living) were counted in the four large cells in the hemocytometer and the percent living cells was calculated.

3. Results

3.1 Effects of H₂O₂ on L-[³⁵S]-cystine uptake and L-[³H]-glutamate uptake

As previously mentioned, the expression of System x_c⁻ is regulated by the redox state of the cell (Bannai, 1984; Miura et al., 1992; Ruiz et al., 2003; Sato et al., 2001). Recent *in vitro* and *in vivo* studies have demonstrated that System x_c⁻ activity is induced within hours of exposure to oxidative agents (32) or onset of hypoxia/ischemia (Fogal et al., 2007; Mysona et al., 2009). However, the acute regulation of transporter activity by oxidative agents has not been studied. Therefore, we sought to determine if a brief exposure of oxidants to cultured cells could trigger a rapid change in System x_c⁻ activity independent of an increase in transporter expression.

We found a 10 min exposure of confluent cultured human glioma cells (U-138MG) to 0.3 mM H₂O₂ resulted in an immediate three-fold increase in ³⁵S-L-cystine transport ($t(22)=-3.81$, $p<0.001$), with no significant change in the non-specific cystine transport (Figure 2A; $t(17)=0.12$, $p=0.9$). This effect of H₂O₂ was dose dependent with an EC₅₀ of 65 μ M H₂O₂ (Figure 2B) and did not return to baseline until 120 min following removal of the oxidant from the medium (Figure 2C). Kinetic analysis of cystine transport indicated that 0.3 mM H₂O₂ caused a nearly two-fold increase in the V_{max} of cystine transport (from 297 ± 58 pmols/mg protein/min to 513 ± 58 pmols/mg protein/min), with a little change in the observed K_m (100 μ M compared to 117 μ M, respectively, Figure 2D).

3.2 Effects of H₂O₂ on cell surface expression of System x_c⁻

The fact that we observed a doubling of the V_{max} of ³⁵S-L-cystine transport within ten minutes of H₂O₂ exposure suggests that H₂O₂ 1) rapidly increases the rate of *de novo* synthesis of xCT, 2) increases the cell surface expression of the System x_c⁻ and/or 3) modifies the transporter in such a way to increase its basal catalytic rate. Given that several transporters, including the excitatory amino acid transporters (EAATs) (Robinson, 2006; Santos et al., 2009), monoamine transporters (Melikian, 2004), GABA transporters (Wang and Quick, 2005) and glucose transporter (GLUT4) (Hou and Pessin, 2007), are known to undergo rapid regulated membrane trafficking to and from the cell surface in response to various

stimuli, we sought to test the hypothesis that H₂O₂ triggers the translocation of System x_c⁻ from intracellular compartments to the plasma membrane.

System x_c⁻ belongs to the family of HAAT proteins that includes nine different members with unique amino acid substrate specificities (for review see Ye et al., 1999). Like most members of the HAAT family, System x_c⁻ is composed of two proteins, a 50 kD light chain protein (xCT) that is linked through a disulfide bond to a 98 kD type II glycosylated heavy chain protein, 4F2HC (also named CD98hc, FRP-1, and SLC3A2) (Sato et al., 2000, 1999). Since the xCT light chain confers the transport activity of System x_c⁻, we first examined the membrane localization of xCT in U-138MG cells using a cell surface biotinylation assay. In confluent U-138MG cells, only 34 ± 3% of xCT is localized to the plasma membrane (Figure 3A), similar to other transport systems that undergo regulated trafficking to and from the cell surface (Fournier et al., 2004; Loder and Melikian, 2003; Staub et al., 2000; Wang and Quick, 2005). After exposure of glioma cells to 0.3 mM H₂O₂ for 10 min, however, there is a statistically significant shift in xCT localization to the plasma membrane ((Figure 3A and 3C; t(14)=-4.072; p<0.001)). The total xCT immunoreactivity measured in the lysate fraction does not increase as a result of H₂O₂ treatment (1.07 ± 0.15-fold change compared to control (t(7)=0.472; p=0.651)) confirming that H₂O₂ does not increase the *de novo* expression of xCT. In addition, actin is not visible in the biotinylated fraction in H₂O₂ treated samples, demonstrating that the H₂O₂ treatment does not compromise the integrity of the membrane (Figure 3A).

Previous studies have suggested that 4F2HC functions to regulate the trafficking of light chains which belong to the HAAT family. Therefore, we sought to measure the changes in 4F2HC trafficking in response to H₂O₂. Similar to xCT, a small fraction of 4F2HC is observed on the membrane in confluent U-138MG cells, however, 4F2HC does not undergo a comparable shift to the membrane in H₂O₂ treated cells (Figure 3B and 3C; t(6)=-0.235; p=0.822)). Thus, our data suggest that either H₂O₂ does not trigger a shift in membrane localization of 4F2HC following H₂O₂ or that only a small fraction of 4F2HC traffics to the membrane in response to H₂O₂ which is below the detection limit of our biotinylation assay.

3.3 Effect of H₂O₂ on the trafficking of xCT

Many plasma membrane proteins have been shown to recycle rapidly on the plasma membrane including, but not limited to the GLUT4, epithelial sodium channel (ENaC), dopamine transporter (DAT), transferrin receptor (TfR), and the neuronal Na⁺-dependent glutamate transporter (EAAC1) (Butterworth et al., 2009; Fournier et al., 2004; Hou and Pessin, 2007; Loder and Melikian, 2003; Trowbridge, 1991). The steady state level of a plasma membrane protein is controlled by 1) the rate of its delivery (from either the biosynthetic pathway or recycling endosomes) to the plasma membrane and 2) the rate of its recovery (through the process of endocytosis) from the plasma membrane. Therefore, we sought to determine

whether the H₂O₂-induced rise in membrane localization of xCT was a result of an increase in the rate of xCT delivery to the plasma membrane and/or a decrease in the rate of its removal from the plasma membrane. First, we examined the rate of constitutive delivery of xCT to the plasma membrane in the absence of H₂O₂ using the methods previously established to measure the rate of EEAC1, GAT and TfR delivery (Fournier et al., 2004; Wang and Quick, 2005). Specifically, we treated U-138MG cells with a membrane impermeable biotinylating reagent for increasing lengths of time at 37°C (trafficking permissive conditions) which allows those proteins cycling to the membrane surface to be biotinylated. Following purification of the biotinylated fraction and Western blot analysis, we compared the fraction of biotinylated xCT at 37°C to the fraction of biotinylated xCT at 4°C (trafficking impermissive conditions). We demonstrated that delivery of xCT is dependent on time (One-way ANOVA; $F(3,12)=5.6$, $p=0.013$), with the proportion of xCT labeled at the cell surface doubling in approximately 30 min (Figure 4A). This rate of trafficking of xCT to the plasma membrane is slightly slower than has been observed for EEAC1 and GAT which show doubling rates of 15 min and 5 min, respectively (Fournier et al., 2004; Wang and Quick, 2005). We then used the same biotinylation technique to measure the effect of peroxide exposure on the rate of xCT delivery to the plasma membrane. We discovered that a 10 min exposure to 0.3 mM H₂O₂ leads to a modest, yet significant increase in the fraction of xCT that is biotinylated during this time frame. Specifically, after 10 min, there is a $131 \pm 9\%$ increase in biotinylated xCT relative to the 4°C control, but in the presence of 0.3 mM H₂O₂, there is a $160 \pm 13\%$ increase in biotinylated xCT relative to the 4°C control ($t(6)=-3.57$; $p=0.012$), suggesting that the increase in xCT membrane localization in the presence of H₂O₂ is in part due to an increase in the delivery rate of xCT to the membrane (Figure 4B).

Since we observed an increase in the rate of delivery of xCT to the plasma membrane in the presence of H₂O₂, we next sought to determine the effect of H₂O₂ on the recovery rate of xCT from the plasma membrane using a reversible biotinylation assay. This method requires that most of the biotinylating reagent can be stripped from the protein of interest efficiently using a reducing reagent. Unfortunately, we were unable to strip more than 20 percent of the biotinylating reagent from the cell surface xCT protein with fresh 50 mM GSH, 50 mM MesNA or 50 mM TCEP (data not shown), even when the cells were kept at 4°C (conditions in which trafficking does not occur). Therefore, we used a cell ELISA assay for measuring the internalization rate of xCT (Figure 4B) in the presence and in the absence of H₂O₂. This method has been successfully used to measure internalization of AMPA receptor subunits (Lin et al., 2000; H.-Y. Man et al., 2000), α -adrenergic receptors (Wang et al., 2007), and epidermal growth factor receptors (Johns et al., 2002) in response to various external stimuli. Briefly, cell surface xCT on U-138MG cells was labeled for one hour at 4°C (trafficking impermissive conditions) using a primary antibody directed to an extracellular motif within xCT (goat). Baseline xCT cell surface expression was then measured by applying a secondary antibody coupled to horseradish peroxidase and

subsequent exposure to a chromogenic substrate. To measure internalization rates, cell surface xCT was measured after rewarming the cells to 37°C (trafficking permissive conditions) for 15 min or 30 min after pre-labeling cell surface xCT antibody for one hour at 4°C. The fraction of xCT measured on the membrane in rewarmed samples relative to that measured in samples which were not rewarmed to 37°C is reported in Figure 4C. We demonstrated that xCT undergoes relatively rapid internalization from the membrane at 37°C such that 30 min after cell surface labeling of xCT, only 48% of the labeled xCT remains on the membrane (Figure 4C). When the cells were rewarmed to 37°C for 30 min in the presence of 0.3 mM H₂O₂, however, the internalization rate of xCT decreased significantly, with 82% of the labeled cell surface xCT remaining on the membrane ($t(22) = -4.28$, $p < 0.001$). Thus, while xCT undergoes relatively rapid internalization from the membrane under basal conditions, the rate decreases significantly following H₂O₂ exposure, contributing to an increase cell surface expression of xCT on the plasma membrane.

3.4 Subcellular localization of endogenously expressed xCT in cultured U-138MG cells

Since the trafficking dynamics of xCT appears to be altered in response to H₂O₂, we hypothesized that a significant component of xCT would be localized to early endosomes, critical components of the membrane protein trafficking system. Therefore, we used immunocytochemistry to test the hypothesis that a fraction of xCT is localized to this pool of vesicles. We observed a fraction of intracellular xCT in vesicle-like structures which co-localize with the EEA1 in confluent U-138MG cells (Figure 5), suggesting that xCT does undergo regulated and constitutive trafficking through the endocytic pathway.

In addition to studying the localization of xCT in respect to EEA1, we were interested in examining the co-localization of xCT with 4F2HC. Our biotinylation data suggested that 4F2HC does not exhibit the same H₂O₂-induced increase in cell surface expression as xCT (Figure 3B, C). Therefore, we examined the co-localization of xCT and 4F2HC in U-138MG cells before and after a 10 min exposure to 0.3 mM H₂O₂ (Figure 6A). Overall, there is no effect of H₂O₂ on the observed co-localization (Pearson coefficient) of xCT and 4F2HC in within cells (Figure 6B). However, in vehicle-treated cells, much of the 4F2HC on the membrane and within the cytoplasm does not co-localize with xCT. Instead, the majority of the xCT signal is observed within the intracellular compartment, co-localized with 4F2HC (Figure 6B). This observation is consistent with our hypothesis that only a fraction of the pool of 4F2HC co-localizes with xCT. Following H₂O₂ treatment, there is a greater co-localization of 4F2HC and xCT on the membrane as indicated by an increase in the mean Pearson coefficient around the periphery of the cell ($t(10) = -2.86$, $p = 0.017$; Figure 6C). However, there still remains a pool of 4F2HC localized to the membrane that is not co-localized with xCT. Thus, these data support our earlier suggestion that a small fraction of the total pool of 4F2HC may traffic to the membrane with xCT in response to H₂O₂ treatment.

3.5 Functional Significance of H₂O₂ Regulation of System x_c⁻

Several studies have demonstrated that System x_c⁻ plays an important role in the regulation of intracellular glutathione levels (Cho and Bannai, 1990; Chung et al., 2005; Kato et al., 1992b; Mysona et al., 2009; Ogunrinu and Sontheimer, 2010; Pereira and Oliveira, 1997). In addition, glutathione peroxidase has been suggested to be the most important mechanism for detoxification of H₂O₂ in glioma cells (Dringen et al., 2005). Since this enzyme couples the oxidation of glutathione to the reduction of H₂O₂ to water, the maintenance of glutathione levels is especially important for the detoxification of H₂O₂. Thus, we hypothesized that System x_c⁻ may play a significant role in the maintenance of cellular glutathione levels following an acute exposure to H₂O₂, since under these conditions astrocytes rapidly export glutathione for use by neurons (Wang and Cynader, 2000). To begin to test this hypothesis, we measured total cellular glutathione levels for two hours following a 10 min exposure to 0.3 mM H₂O₂ which has previously been shown to result in the release of intracellular glutathione into the extracellular medium (Hirrlinger et al., 2001; Hohnholt and Dringen, 2014). As expected, H₂O₂ treatment resulted in a rapid decrease in total cellular glutathione which recovered to baseline within 60 min of the removal of the H₂O₂ from the extracellular medium (Figure 7A). Intracellular levels of H₂O₂, however, recovered much more rapidly, nearing baseline levels within a minute of washing the H₂O₂ from the cell culture medium (Figure 7B).

Since we originally observed that H₂O₂-induced changes in System x_c⁻ transporter activity peaked at 30 min, approximately 10 minutes after glutathione levels in the cell reached their lowest level, we hypothesized that the trafficking of xCT may be regulated by either the glutathione or H₂O₂ concentration within the cell. To begin to address this question, we measured the changes in xCT cell surface expression over time following a 10 min exposure to 0.3 mM H₂O₂. We found the H₂O₂-induced increase in xCT membrane expression (Figure 7C) mirrors the time course of System x_c⁻ transporter activity (Figure 2B), and lags slightly behind the recovery of intracellular glutathione levels. Specifically, both xCT cell surface expression and System x_c⁻ transport activity reach a maximum approximately 30 min after the exposure to H₂O₂, and do not return to baseline until 120 min (Figure 4). While these data do not directly address the question as to whether the redistribution of System x_c⁻ to the plasma membrane is necessary to observe the recovery of glutathione levels, the fact that the trafficking event occurs on the same time scale as the observed changes in cellular glutathione levels suggests that the trafficking event does play an important role in the acute regulation of cellular glutathione.

Finally, since we hypothesized that maintenance of cellular glutathione levels is important for protection from oxidative-stress induced cell death, we also assessed the effect of a ten-minute exposure to H₂O₂ on glial cell death. U-138MG cells were grown to 95% confluence and treated with vehicle or 0.3

mM H₂O₂ for 10 min and washed three times with PBS. Twenty-four hours later, the cells were assessed for cell death using the Trypan Blue assay. We observed no difference in the cell survival between vehicle- (89.6% ± 10.1%) and H₂O₂-treated (85.6% ± 12.0%) cells (t(32)=1.062, p=0.296, n=17).

3.6 Recombinant System x_c⁻ expressed in COS-7 cells is also regulated by H₂O₂

System x_c⁻ is highly expressed by human glioma cells (Ye et al., 1999) and is readily induced in many cell types in culture (Devés and Boyd, 2000). Therefore, we wanted to verify that the regulation of endogenously expressed System x_c⁻ by H₂O₂ is not unique to human glioma cells. Therefore, we transiently expressed recombinant myc-xCT and HA-4F2HC in COS-7 cells. We chose this cell line because it does not express xCT endogenously (Figure 1B). Similar to confluent U-138MG cells, we observed that a 10 min exposure of myc-xCT/HA-4F2HC transfected COS-7 cells to 0.3 mM H₂O₂ resulted in a 2.5-fold increase in cell surface expression of myc-xCT (t(6) = -2.618, p=0.04) (Figure 8A, B). In addition, we observed a 1.6-fold increase in HA-4F2HC cell surface expression following H₂O₂ exposure (t(6)= -1.355, p=0.22). While this increase was not statistically significant, these data are consistent with our immunocytochemical analysis (Figure 6) which suggests that xCT:4F2HC heterodimers likely traffic to the membrane in response to oxidants. Finally, these data suggest that the regulation of System x_c⁻ by H₂O₂ is not limited to human glioma cells.

4.0 Discussion

Here we report that the activity of endogenously expressed System x_c⁻ is acutely regulated by exogenous application of H₂O₂ in human glioma cells. Specifically, exposure of cultured human glioma cells to H₂O₂ leads to a three-fold increase in System x_c⁻ activity which is accompanied by a rapid increase in cell surface expression of System x_c⁻. In support of our current findings, Mysona and colleagues (Mysona et al., 2009) previously reported that a six hour exposure of mouse retinal Muller glial cells to oxidative stress through the exogenous application of xanthine and xanthine oxidase led to an 89% increase in System x_c⁻ activity. The increase in activity was the result of a 2.4-fold increase in expression of the mRNA which encodes xCT and a 3.9-fold increase in the cell surface expression of xCT expressed in retinal Muller cells. Similarly, Ogunrinu and colleagues demonstrated that growing D54-MG glioma cells under hypoxic conditions led to a 4-fold increase in the intracellular concentration of reactive oxygen species within 48 hours which was also accompanied by a 3.5-fold increase in cell surface expression of xCT, a 1.5-fold increase in System x_c⁻ activity, and an increase in glutathione utilization (Ogunrinu and Sontheimer, 2010). Similar to what we have observed here, the total expression of xCT was not changed during the hypoxic exposure. While these studies were not focused on the acute regulation of System x_c⁻

trafficking by oxidants, they provide further evidence that oxidative stress regulates the cell surface expression of System x_c^- .

The acute modulation of cell surface expression of many transporters is a common mechanism of functional regulation (for review see Maxfield and McGraw, 2004; Hou and Pessin, 2007; Santos et al., 2009). One of the best studied examples is the regulation of the trafficking of GLUT4 by insulin (Hou and Pessin, 2007). In muscle and adipose tissue, insulin triggers the movement of intracellularly localized GLUT4 to the plasma membrane, thus facilitating the uptake of glucose into cells when glucose levels are high. In kidney epithelial cells, aldosterone and vasopressin increase the cell surface expression of epithelial sodium channel (ENaC) allowing for maintenance of proper sodium balance, blood volume and blood pressure (Butterworth et al., 2009). In neurons and glia, neurotransmitter transporters exhibit reduced cell surface expression following activation of protein kinase C (Melikian, 2004; Robinson, 2006). In each of these examples, the transporters are observed in endosomal compartments within the cell, and the rates of exocytosis and internalization of the transporter are altered. We have shown that System x_c^- is localized to early endosomes within glioma cells and that the increase in cell surface expression is a result of both an increase in the rate of delivery of xCT to the plasma membrane and a decrease in the rate of xCT internalization from the plasma membrane.

The fact that the time-course for the H_2O_2 -triggered increase in transporter activity and cell surface expression correlates with changes in total cellular glutathione levels suggests that the process described here may be a component of a critical feedback mechanism which allows cells to rapidly respond to oxidative insults and increase cellular glutathione levels. Peroxides are produced as a result of aerobic metabolism in all cell types (for review see Dringen et al., 2005). Specifically, superoxide produced during mitochondrial respiration is continuously detoxified to H_2O_2 through the action of the enzyme superoxide dismutase. Once formed, H_2O_2 must be rapidly removed from cells to prevent metal-catalyzed formation of highly reactive hydroxyl radicals that can cause irreversible covalent modification of DNA, lipids, and proteins. H_2O_2 is enzymatically degraded by the action of two enzymes, catalase and glutathione peroxidase (GPx). Since GPx couples the reduction of H_2O_2 to H_2O to the oxidation of glutathione, loss of cellular glutathione can rapidly lead to increases in cellular H_2O_2 (Dringen et al., 2005).

Given that the brain is the most metabolically active tissue, there is increased need for maintenance of glutathione levels for proper regulation of H_2O_2 (Dringen et al., 2005). A microdialysis study in freely moving rats established that the baseline concentration of H_2O_2 in the extracellular fluid in the striatum is about 25 μM (Hyslop et al., 1995). However, within 20 min after a brief ischemic episode (30 min), the extracellular H_2O_2 concentration increases to 150 μM before recovering to baseline levels one hour later. Given that the EC_{50} we observed for H_2O_2 -induced increase in System x_c^- activity is at

least-two fold lower (65 μ M), suggests that the regulation of System x_c^- cell surface trafficking is physiologically relevant, and may serve to increase cellular reserves of cystine for glutathione synthesis. In support of this hypothesis, Soria and colleagues recently observed a large increase in System x_c^- activity in rodent cerebral cortex organotypic cultures within 12 minutes of onset of a 45 min oxygen glucose deprivation (Soria et al., 2014). Using PET imaging, the same group also demonstrated that System x_c^- activity increases *in vivo* in the rat striatum 1.5-fold within five minutes of reperfusion following two hours of transient focal ischemia. System x_c^- activity continued to stay elevated for at least five hours following reperfusion. While the investigators were unable to measure System x_c^- activity in the rats during the transient ischemic event, our data suggests that the upregulation of the transporter activity may actually occur during the onset of the ischemic event when oxidant levels rise rapidly in the brain. Collectively, the data from these studies are consistent with the hypothesis that ischemic events lead to rapid increases in H_2O_2 levels which trigger an increase in cell-surface expression of System x_c^- .

Whether the H_2O_2 -mediated increase in cell surface expression of System x_c^- is actually driven by H_2O_2 or by the immediate drop in intracellular glutathione that accompanies the oxidative stimulus cannot be directly determined from this study. Similarly, we cannot ascertain from our data whether it is intracellular H_2O_2 or extracellular H_2O_2 which triggers the trafficking of xCT. We did demonstrate that H_2O_2 levels rise intracellularly after we applied it extracellularly, but we did not whether it was intracellular or extracellular H_2O_2 (or both) that triggered the translocation. Similar doses of exogenously applied H_2O_2 have previously been shown to increase the cell surface localization of GLUT4 in adipocytes (Mahadev et al., 2001) and cardiac myocytes (Horie et al., 2008) and ENaC in alveolar epithelial cells (Downs et al., 2013). H_2O_2 is also known to activate several cellular signaling cascades (Truong and Carroll, 2013) including, but not limited to, phosphoinositol-3-kinase(PI3K)/Akt Kinase, AMP-activated protein kinase (AMPKinase), mitogen activated protein kinase (MAPK), and several Receptor Tyrosine Kinases (RTKs), many of which have been shown to regulate the cell surface expression of several transporters and proteins. Thus, it is plausible that H_2O_2 triggers the activation of one or more signaling pathways leading to a transient increase in cell surface expression of xCT in a manner analogous to that observed for GLUT4 and ENaC. However, we also observed the time course of increased System x_c^- activation and xCT cell surface expression lags slightly behind the rate of recovery of intracellular glutathione levels, while intracellular H_2O_2 levels returned closer to baseline within min following wash of H_2O_2 from the extracellular compartment. Therefore, it is plausible that the trafficking of xCT may be regulated by intracellular glutathione levels itself.

Despite the fact that we observed a significant increase in the cell surface expression of xCT in the presence of H_2O_2 , we were not able to detect a comparable change in the cell surface expression of endogenously expressed 4F2HC in cultured glioma cells. Previous studies have suggested that 4F2HC

regulates the trafficking of the light chain proteins of the HAAT family, thus, we expected to observe a shift in the cell surface expression of 4F2HC during H₂O₂ exposure (Verrey et al., 2004). Instead, we observed that the majority of 4F2HC was found within the intracellular compartment, with no statistically significant increase following H₂O₂ exposure. Given that 4F2HC forms dimers with multiple HAAT protein light chains and β -integrins (Cantor and Ginsberg, 2012; Devés and Boyd, 2000), the absence of an observed shift of 4F2HC to the membrane in response to H₂O₂ does not specifically demonstrate that xCT traffics independently of 4F2HC under these conditions. It may be that our membrane biotinylation assay is not sensitive enough to detect the fraction of the total amount of 4F2HC that actually shifts to the membrane in response to H₂O₂, given that only a small fraction of 4F2HC is expected to be bound to and trafficking with xCT. This possibility is strengthened by our observations that 1) a significant component of the intracellular 4F2HC immunoreactivity we observed in human glioma cultures did not co-localize with xCT in both control and H₂O₂ conditions (Figure 6B) and 2) recombinant HA-4F2HC expressed in COS-7 cells showed a greater tendency to traffic to the membrane with myc-xCT in response to H₂O₂ (Figure 7A, B). However, these data do suggest that the H₂O₂-mediated increase in xCT cell surface expression is a result of inherent regulation of the xCT protein, and not 4F2HC.

The fact that we have observed acute oxidant-mediated regulation of endogenously expressed System x_c⁻ in U-138MG cells and recombinant myc-xCT and HA-4F2HC expressed in COS-7 cells suggests that this may be a universal defense mechanism for rapid manipulation of glutathione levels in response to oxidative insult. The chronic regulation of System x_c⁻ by oxidants and glutathione levels has been studied in numerous cell types including activated macrophages, microglia, fibroblasts, cultured neurons, astrocytes (for review see Bridges et al., 2012), and these studies have demonstrated that xCT is transcriptionally activated under conditions of oxidative stress leading to a 2-3 fold increase in xCT expression level. We now suggest that xCT will also exhibit a greater cell surface localization under these same conditions, thus leading to additional stimulation in System x_c⁻ activity beyond that achieved by an increase in xCT synthesis alone.

The increased capacity for glutathione production in astrocytes in response to oxidative stress also has significant implications for neuronal survival given the central role that astrocytes play in regulating neuronal glutathione levels (For review, see McBean, 2017). Unlike astrocytes, neurons do not rely on System x_c⁻ for the import of cystine to supply a source of cysteine for glutathione production. Instead, neurons directly import cysteine through the excitatory amino acid carrier-1. The source of the cysteine, however, is astrocytic glutathione, which is maintained in relatively high concentrations (up to 8 mM). Specifically, astrocytes export glutathione through the multidrug resistance protein-1 where it is degraded by γ -glutamyl transpeptidase and neuronal dipeptidase into γ -glutamate, cysteine and glycine. The cysteine and glycine are imported into neurons and used for glutathione synthesis, while the γ -glutamate is

recycled back into astrocytes. Thus, under conditions of oxidative stress, glutathione export from astrocytes increases, as well as the demand for glutathione production to re-establish baseline glutathione levels. Therefore, our observations that System x_c^- exhibits increased activity immediately in response to H_2O_2 exposure and glutathione export in astrocytoma, suggests that the increased capacity for cystine uptake and glutathione production in astrocytes may be important for both astrocytic and neuronal oxidative balance.

While an increase in System x_c^- -mediated cystine uptake likely helps to ameliorate oxidative stress, it is important to note that the associated obligatory upsurge in glutamate release by the transporter will likely have a significant impact on extracellular glutamate levels and neighboring neurons. Since, System x_c^- contributes to 60% of the total extracellular glutamatergic pool in the brain (D. A. Baker et al., 2002; David A Baker et al., 2002), it is not surprising increases in System x_c^- activity are associated with 1) activation of extra-synaptic metabotropic glutamate receptors which regulate vesicular neurotransmitters release, 2) changes in the induction of long-term potentiation and long-term depression, and 3) alterations in synaptic strength (D. A. Baker et al., 2002; Moran et al., 2005, 2003; Moussawi et al., 2011, 2009). In fact, Fogal and colleagues (Fogal et al., 2007) demonstrated that hypoxia induces excitotoxicity in mixed cortical-cell cultures in a System x_c^- -dependent manner. Thus, while oxidant-mediated increases in System x_c^- cell surface expression might function to combat the developing oxidative stress associated with ischemic events, it likely will contribute to a rise in glutamate release from astrocytes. Such upregulation of System x_c^- is observed in human gliomas, resulting in epileptic seizures, excitotoxic cell death of neurons, and rapid expansion of the tumor (Buckingham et al., 2011; Savaskan et al., 2008; Ye et al., 1999).

5.0 Conclusions:

In summary, this is the first report that the cell surface expression of System x_c^- can be acutely regulated by oxidants. This effect is dose dependent with an EC_{50} of 65 μM and persists for nearly 60 min following the removal of the stimulus. In addition, the oxidant-induced change in activity and cell surface expression is consistent with the time-course of cellular glutathione recovery, suggesting we have identified a novel positive feed-back loop wherein redox stress leads to an upregulation of System x_c^- activity and an increase in available cystine (and cysteine) for glutathione synthesis. Given the unique role of System x_c^- in regulating both the redox environment and extracellular glutamate levels and its implication in the pathogenesis of stroke, addiction, schizophrenia, macular degeneration, Alzheimer's Disease and Parkinson's Disease (Bridges et al., 2012), it is imperative that future studies are aimed at developing a more comprehensive understanding of the processes which regulate System x_c^- trafficking.

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Figure Captions

Figure 1. Characterization of xCT antibody for use in western blot and immunocytochemical analysis.

A. Control U-138MG or DEM-treated U-138MG cells were immunoblotted for xCT and actin (loading control). Note a 40 kDa band for xCT under control conditions (lane 1) and 2 bands at 27 kDa and 40 kDa following a 24-hour treatment with DEM. B and C. COS-7 cells transfected with either the myc-xCT pCMV-3Tag-2A construct (4 μ g or 2 μ g of DNA) or the empty pCMV vector (4 μ g) were immunoblotted for xCT and anti-actin (B) or myc (C). N.S. labels a non-specific band that is visualized with the anti-myc antibody only in the membrane fraction (M) but not in the intracellular fraction (I) when performing cell surface biotinylation assays. D. COS-7 cells transfected with myc-xCT pCMV-3TAG-2A immunostained for c-myc (red) and xCT with the novel anti-xCT antibody (green). Pearson correlation of 0.972.

Figure 2. H₂O₂ triggers an increase in System x_c⁻ activity in cultured U-138MG cells. A. Mean uptake ($\pm$ S.E., n=12) of ³⁵S-L-cystine (20 μ M) for 10 min in U-138MG cells treated with either 0.3 mM H₂O₂ or vehicle for 10 min. CPG (0.2 mM) was used to identify System x_c⁻-specific cystine uptake (*p=0.001). B. Dose dependence of the effect of H₂O₂. Mean specific ($\pm$ S.E., n=6) ³⁵S-L-cystine transport activity cystine (25 μ M) measured 10 min following exposure to various concentrations of H₂O₂. The data were fit to the equation ($y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(\text{LogEC}_{50} - x)})$; $R^2 = .9945$) giving an EC₅₀ for H₂O₂ of 65 μ M. C. Mean specific ³⁵S-L-cystine transport activity ($\pm$ S.E., n=4) measured at various time points following the 10 min exposure of cells to 0.3 mM H₂O₂ (indicated by black bar). There was a significant effect of time on transport activity ($F(6,29) = 23.2$, $p < 0.001$, * indicates data significantly different than time 0 $p < 0.05$). D. Steady state kinetic analysis of cystine transport following a 10 min exposure to 0.3 mM H₂O₂ or vehicle (control) for 10 min. The data are fit to the hyperbolic function $\text{uptake} = V_{\text{max}} * [\text{cystine}] / (K_m + [\text{cystine}])$. Under control conditions $V_{\text{max}} = 297$ pmols/mg protein and $K_m = 100$ μ M. After a 10 min exposure to 3 mM H₂O₂, $V_{\text{max}} = 513$ pmols/mg protein/min and $K_m = 117$ μ M. (n=6 $\pm$ S.E.).

Figure 3. Trafficking of endogenously expressed xCT and 4F2HC in U-138MG cells in response to H₂O₂ exposure. Biotinylation assays were used to label cell surface xCT (A) and 4F2HC (B) in cultured U-138MG cells exposed to either 0.3 mM H₂O₂ or vehicle for 10 min. xCT in lysate (L), intracellular (I) and membrane (M) fractions were visualized by western blot. The integrity of the biotinylation assay was determined by probing the same blots with anti-actin. The mean percentage of xCT and 4F2HC found on the cell surface is plotted (n=8; *p=0.001) in C.

Figure 4. Effect of H₂O₂ on constitutive delivery and recovery of xCT from the plasma membrane.

A. Mean rate of constitutive delivery of endogenously expressed xCT to the plasma membrane was measured over time. Data are presented as percentage of xCT immunoreactivity observed in the biotinylated (B), non-biotinylated (NB) or total xCT in cell lysate (T) under trafficking-impermissive conditions (4°C). There is a significant effect of time on the percent of xCT that is found in the biotinylated fraction, i.e. there is an effect of time on the delivery of xCT to the membrane ($F(3,12)=5.56$, $p=0.013$, $n=4 \pm \text{S.E.}$). B. Mean fraction of biotinylated xCT following a 10 min exposure of U-138MG cells to vehicle (control) or 0.3 mM H₂O₂ ($n=4 \pm \text{S.E.}$; $*p=0.01$). C. Cell surface internalization rates of endogenously expressed xCT were measured using a colorimetric cell ELISA-based assay. Cell surface xCT was labeled in cultured U-138MG cells for one hour at 4°C with a goat anti-xCT antibody directed to an epitope in the extracellular region of the protein. The total labeled xCT was measured after warming the cells to 37°C for either 15 min or 30 min in the presence and absence of 0.3 mM H₂O₂ and compared to the total labeled xCT in samples not warmed to 37°C ($t=0$). Data are expressed as percent of total surface expression at $t=0$ ($n=12 \pm \text{S.E.}$; $**p=0.001$).

Figure 5. Subcellular localization of endogenously expressed xCT in U-138MG cells. xCT (green) and EEA-1 (red) were visualized in cultured U-138MG cells. Co-localization is shown in the third panel (merge) and the area within the white box is shown in the fourth panel.

Figure 6. H₂O₂-induced changes in subcellular localization of endogenously expressed xCT and 4F2HC in U-138MG cells. A. xCT (green) and 4F2HC (red) were visualized in cultured U-138MG cells treated with either 0.3 mM H₂O₂ or vehicle (control). Co-localization is shown in the third panel (merge) and the Pearson coefficients for the cell is given in white. The area within the white box is shown in the fourth panel and white arrows indicate 4F2HC immunostaining (red) which does not co-localize with xCT immunostaining (green). B. The green and red fluorescence intensity is plotted as a function of pixel number along a cross-section (indicated by the yellow arrows in A) of control and H₂O₂-treated cells. C. Pearson coefficients were measured for six cells from three separate experiments under both control and H₂O₂ conditions. In addition, the Pearson coefficients were also measured for the periphery (cell membrane) by identifying a ROI 20-pixels in width along the outer edge of the cells ($*p=0.017$).

Figure 7. Time course of changes in total cellular glutathione, xCT cell surface expression and intracellular H₂O₂ levels following a 10 min H₂O₂ exposure A. Mean total cellular glutathione levels ($n=6 \pm \text{S.E.}$) after a 10 min exposure to either 0.3 mM H₂O₂ (indicated by black bar) or vehicle (control) are plotted as a function of time. * Indicates those data which are significantly different from control

p<0.05. B. Mean intracellular H₂O₂ levels (n=24 ± S.E) in U13MG were plotted as a function of time following either a 10 min exposure to 0.3 mM H₂O₂ (indicated by black bar) or vehicle. C. Mean percent of xCT (n=4 ± S.E) localized to the plasma membrane is plotted over time following a 10 min exposure to 0.3 mM H₂O₂ (indicated by black bar). Percent xCT on membrane is calculated by dividing the immunoreactivity measured in the membrane (biotinylated) fraction by the total xCT immunoreactivity (intracellular (I) + membrane (M)). *Indicates those data which are significantly different than time 0, p<0.05.

Figure 8. H₂O₂-induced trafficking of transiently-expressed, recombinant myc-xCT and HA-4F2HC in COS-7 cells. A. myc-xCT expressed in COS-7 cells exhibits a shift from the intracellular (I) compartment to the membrane (M). The trafficking of HA-4F2HC is less pronounced. N.S. labels the non-specific band that is visible only in the intracellular compartment. B. H₂O₂ exposure has a significant effect on mean relative membrane expression of myc-xCT, but not HA-4F2HC. n=4, *p<0.05

D

Figure 1

Figure 2

Figure 3

A **B** **C** **Figure 4**

A**U-138MG** **Figure 5**

A**Control** **H₂O₂** **B** **C** **Figure 6**

Figure 7

A **B** **Figure 8**