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Expression of the glutamate-cystine (x_c^-) exchanger in cultured retinal ganglion cells and regulation by nitric oxide and oxidative stress

Ying Dun¹, Tracy Van Ells¹, Barbara Mysona¹, M. Shamsul Ola¹, Vadivel Ganapathy², and Sylvia B. Smith^{1,3,*}

¹Department of Cellular Biology and Anatomy, Medical College of Georgia, Augusta, GA

²Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA

³Department of Ophthalmology, Medical College of Georgia, Augusta, GA

Abstract

Purpose—The cystine-glutamate transporter, system x_c^- , mediates the Na^+ -independent exchange of cystine into cells coupled to the efflux of intracellular glutamate. Within cells, cystine is reduced to cysteine, a component of glutathione, thus system x_c^- plays a critical role in glutathione homeostasis. Studies in brain had initially suggested that system x_c^- was present primarily in astrocytes and not neurons, but more recent work suggests that certain brain neurons have an active system x_c^- . In the retina, system x_c^- has been demonstrated in retinal Müller and RPE cells. Recent immunohistochemical work from our lab suggested that the two protein components of system x_c^- , xCT and 4F2hc, are present in ganglion cells of the intact retina. The purpose of this investigation was to determine whether system x_c^- was present in primary ganglion cells isolated from neonatal mouse retinas and, if so, to study its regulation by oxidative stress in a retinal ganglion cell line, RGC-5.

Methods—The presence of xCT and 4F2hc in RGC-5 cells was established by RT-PCR, immunoblotting and immunohistochemistry and in primary mouse retinal ganglion cells by immunoblotting and immunohistochemistry; the function of the transporter in RGC-5 cells was established by measuring radiolabeled glutamate uptake in the absence of Na^+ . To assess regulation of system x_c^- by oxidative stress in ganglion cells, RGC-5 cells were incubated in the presence or absence of nitric oxide (NO) donors (3-nitroso-N-acetylpenicillamine (SNAP), S-nitrosoglutathione (SNOG), or 3-morpholiniosydnonimine hydrochloride (SIN-1) and reactive oxygen species (ROS) donors menadione sodium bisulfite, hydrogen peroxide and xanthine sodium salt/xanthine oxidase and system x_c^- was analyzed using functional assays, RT-PCR and immunoblotting.

Results—RGC-5 cells and primary ganglion cells isolated from mouse retina express xCT and 4F2hc as demonstrated by RT-PCR, immunoblotting and immunohistochemistry. RGC-5 cells take up glutamate in the absence of Na^+ and this uptake was blocked by known inhibitors of system x_c^- , glutamate, cysteine, and cystine as well as quisqualic acid. Treatment of RGC-5 cells with NO donors and donors of ROS led to an increase in the functional activity of system x_c^- . Kinetic analysis of SNAP-treated RGC-5 cells compared to control cells showed that the increase was associated with an increase in the maximal velocity of the transporter with no significant change in the substrate affinity. Molecular analyses showed that the upregulation is associated with an increase in the expression of xCT with no detectable change in the expression of 4F2hc.

* Please send correspondence to: Sylvia B. Smith, Ph.D., Department of Cellular Biology and Anatomy, Medical College of Georgia 1459 Laney-Walker Blvd., CB 2820, Augusta, GA 30912-2000, 706-721-7392 (phone), 706-721-6839 (fax), sbsmith@mail.mcg.edu.

Conclusions—RGC-5 cells and ganglion cells isolated from neonatal mice express the cystine/glutamate transporter x_c^- (the light chain xCT and the heavy chain 4F2hc) as is evident from functional and molecular studies. Oxidative stress upregulates this transport system in RGC-5 cells and the process is associated with an increase in xCT mRNA and protein but no change in 4F2hc mRNA or protein.

Keywords

oxidative stress; retinal ganglion cells; cystine/glutamate transporter; nitric oxide

Introduction

The cystine-glutamate transporter, known as system x_c^- , mediates the Na^+ -independent, electroneutral exchange of cystine and glutamate [1,2]. System x_c^- is a member of the glycoprotein-associated amino acid transporter (gpaAT) family [3]. Transporters in this family are obligate exchangers that form heterodimers with a glycoprotein subunit, called the heavy chain, permitting functional surface expression. System x_c^- is comprised of the transporter subunit xCT, a 502 amino acid containing protein with twelve putative transmembrane domains, and the heavy chain 4F2hc (also known as CD98), a cell surface glycoprotein.

Under physiological conditions, system x_c^- transports cystine into cells coupled to the efflux of intracellular glutamate. Once inside the cell, cystine is reduced to cysteine, which is required for synthesis of the antioxidant, glutathione (GSH), a tripeptide consisting of glutamic acid, cysteine, and glycine. Indeed, cellular synthesis of GSH is limited by intracellular levels of cysteine, thus system x_c^- plays a critical role in GSH homeostasis. As GSH is necessary for protection of cells against oxidative damage, system x_c^- assumes significance as a transport system closely associated with the cellular antioxidant machinery. The relevance of system x_c^- to cellular antioxidant processes is underscored by its upregulation in multiple cell types exposed to oxidative stress [4-9].

As reviewed by McBean [2], experimental evidence supports a model in which cystine is taken up by astrocytes for GSH formation, which is then released to provide precursors for neuronal GSH. The model is based on early observations that neurons did not accumulate cystine well, however new evidence has emerged from studies of both hippocampal [10] and cortical neurons [11], demonstrating uptake of cystine. Thus, though astrocytes appear to be important in providing GSH precursors to neurons, these recent results suggest that at least some neuronal cells in culture are able to efficiently transport cystine, and maintain adequate GSH levels even without astrocytic support [10, 12].

The retina is extremely vulnerable to oxidative stress and it is not surprising that system x_c^- has been studied in this tissue. Work by Kato in the early 1990's [13] described system x_c^- expression in Müller cells, the predominant glial cell of the retina. These findings were confirmed by Pow in 2001 [14] using an antibody against aminoadipic acid, which is a selectively transported substrate for system x_c^- . System x_c^- has been studied extensively at the functional and molecular level in Müller cells [15] and also in retinal endothelial cells [7,8]. In addition to these two cell types, our laboratory characterized system x_c^- in retinal pigment epithelial (RPE) cells and showed that it is upregulated by exposure to nitric oxide donors [4] as well as by the HIV Type 1 transactivator protein, Tat [16]. In this latter study, we performed immunolocalization studies with affinity purified antibodies produced in our laboratory against xCT and with commercially available antibodies against 4F2hc (CD98). These studies suggested colocalization of these components of system x_c^- in several retinal cell layers, including neurons of the ganglion cell layer. We were intrigued that system x_c^-

might be present in retinal ganglion cells. These cells are the second order neurons of the visual system and are susceptible to many types of insult including oxidative stress [17,18] and die in a number of retinal diseases such as glaucoma [19]. Recently, we and others have reported that they die in diabetic retinopathy [20-22]. Nitric oxide (NO) is a molecule implicated in the pathogenic complications of diabetic retinopathy [23-25]. Yilmaz and co-workers [26] reported a fivefold elevation of NO in the vitreous of patients with proliferative diabetic retinopathy compared with nondiabetic control patients. Given that NO levels are increased in diabetic retinopathy [23-26], coupled with our findings that NO can upregulate system x_c^- transporter in RPE cells [4], we asked whether this transporter is present in ganglion cells and if so, whether it is upregulated by NO in these cells.

In the present study, we used the retinal ganglion cell line (RGC-5) [27] to assess the effects of various NO donors on the activity and expression of the system x_c^- . Since this is a transformed cell line, we also studied primary ganglion cells isolated from neonatal mouse retinas and determined that xCT was indeed present in these primary neurons. Using the RGC-5 cells, we demonstrate that exposure of cells to the NO donor SNAP leads to a significant increase in system x_c^- activity and we demonstrate that exposure of these cells to SNAP upregulates the gene expression of the xCT component of system x_c^- . Additional experiments with various other donors of reactive oxygen species show similar stimulation of system x_c^- in the RGC-5 cells.

Materials and Methods

Reagents

Reagents used in this study were obtained from the following sources: Dulbecco's modified Eagle's medium F12 (DMEM:F12) medium, Earl's Balanced Salt Solution, Dulbecco's Phosphate Buffered Saline, Neurobasal medium, TRIzol reagent, penicillin-streptomycin (Gibco-Life Technologies, Rockville, MD); [2,3- ^3H]-glutamate (specific radioactivity 50.0 Ci/mmol) (Amersham Bioscience, Arlington Heights, IL), L-[4,5- ^3H]-leucine (specific radioactivity 60 Ci/mmol) (New England Nuclear, Boston, MA), L-[2,3- ^3H]-alanine (specific radioactivity 40 Ci/mmol) (Moravsek Biochemicals, Brea, CA); 4F2hc (CD98) goat polyclonal IgG, goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Inc, Santa Cruz, CA); Cy-3 conjugated anti-rabbit IgG, peroxidase-conjugated AffiniPure rabbit anti-goat IgG, Alexa Fluor 488-conjugated anti-goat IgG (In Vitrogen, Carlsbad, CA); Hoeschst dye 33342 (Molecular Probes, Eugene, OR); ECL detection kit (Pierce Biotechnology, Inc., Rockford, IL); Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA); Power Block (BioGenex, San Ramon, CA); Glutathione (GSH) Detection Kit (Chemicon International, Temecula, CA); complete Mini Protease Inhibitor Cocktail tablets, GeneAmp RNA PCR Kit (Applied Biosystems/Roche Molecular System, Branchburg, NJ); TaKaRa TaqTM (Takara Bio Inc, Otsu, Shiga, Japan); sense and antisense primers for xCT and 4F2hc (Integrated DNA Technologies, Inc, Coralville, IA); papain (Worthington, Lakewood, NJ), trypsin inhibitor (Roche Applied Science, Mannheim, Germany), rabbit anti-macrophage antiserum (Accurate Chemical Corp., Westbury, NY), AffiniPure donkey anti-rabbit IgG (H+L) (Jackson, ImmunoResearch, Westgrove, PA), purified rat anti-mouse Thy 1.2 antibody (Pharmingen, San Diego, CA), brain-derived neurotrophic factor, ciliary-derived neurotrophic factor (basic fibroblast growth factor (PeproTech Inc., Rocky Hill, NJ), 3-nitroso-N-acetylpenicillamine (SNAP), S-nitrosoglutathione (SNOG), 3-Morpholiniosydnonimine hydrochloride (SIN-1), menadione sodium bisulfite, hydrogen peroxide 30% (W/W) solution, xanthine sodium salt, xanthine oxidase, fetal bovine serum, monoclonal antibody to β -actin and all other chemicals (Sigma-Aldrich Chemical Co., St. Louis, MO); RGC-5 cells were the kind gift of Dr. Neeraj Agarwal, Department of Anatomy and Cell Biology, University of North Texas Health Science Center, Fort Worth, TX.

Culture of RGC-5 cells

The development of the rat retinal ganglion cell line (RGC-5) has been described [27, 18]. These cells express markers for ganglion cells such as Thy-1, Brn-3C, neurotrophins and their cognate receptors, but they are negative for markers of Müller, amacrine, and horizontal cells suggesting they represent a ganglion cells. The cells were maintained at 37°C in a humidified chamber of 5% CO₂. They were cultured in 75 cm² flasks with Dulbecco's modified Eagle's medium:nutrient mixture F12 (DMEM:F12), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. The culture medium was replaced with fresh medium every other day. Upon confluency, cultures were passaged by dissociation in 0.05% (w/v) trypsin in 0.01M phosphate-buffered saline (PBS).

Isolation and culture of primary mouse ganglion cells

C57BL/6 mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN), were maintained in our colony, as described previously [22]. They were bred in our animal facilities following our IACUC approved protocol. Mouse retinal ganglion cells were isolated following the method of Barres et al. [28] with minor modifications. Briefly, retinal tissue was dissected from 2-day-old C57BL/6 mice. Twelve retinas were combined and incubated for 30 min at 37 °C in Dulbecco's Phosphate Buffered Saline (DPBS) containing 16.5 units/ml papain, 0.2mg/ml N-acetyl-L-cysteine and 0.04% DNase. Tissues were triturated in DPBS containing 0.15% trypsin inhibitor, 0.15% bovine serum albumin (BSA), and rabbit anti-macrophage antiserum until retinas were dissociated. Cells were pelleted by centrifugation for 5 min at 200×g then suspended in DPBS containing 1% trypsin inhibitor and 1% BSA. The retinal cell suspension was centrifuged at 200×g for 5 min, and the pellet was resuspended in panning buffer (DPBS, 0.02% BSA, 5 µg/ml insulin). Macrophages and microglial cells were removed by incubation of the cell suspension for 40 min at room temperature on a panning plate (75 ml flask) coated with AffiniPure donkey anti-rabbit IgG (H+L)). The non-adherent cells were incubated for 1 h at room temperature on a second panning plate (100-mm Petri dish) coated with affiniPure donkey anti-rat IgG (H+L) antibody and purified rat anti-mouse Thy 1.2 antibody. The panning dish was washed 4-5 times with DPBS. Adherent cells were released from plate by incubation with 0.125% trypsin in Earl's Balanced Salt Solution for 8 min at 37 °C. Fetal bovine serum (30%) in Neurobasal medium was added to the suspension, and ganglion cells were collected by centrifugation at 200×g for 10 min. The pellet was then resuspended in base medium (Neurobasal medium containing 5µg/ml insulin, 1 mM sodium pyruvate, 0.1 mg/ml transferrin, 60 ng/ml progesterone, 16 µg/ml putrescine, 40 ng/ml sodium selenite, 40 ng/ml triiodothyronine, 1mM L-glutamine, 60 µg/ml N-acetyl cysteine, 2% B27, 50 ng/ml brain-derived neurotrophic factor, 10 ng/ml ciliary-derived neurotrophic factor, 10 ng/ml Forskolin, 10 ng/ml basic fibroblast growth factor, 0.1 mg/ml bovine serum albumin). Cells were grown on poly D-lysine and laminin-coated coverslips. Half of the media was changed every third day. Cells were subjected to immunocytochemistry after three days of maintenance.

Measurement of system x_c⁻ activity

Since system x_c⁻ is an exchanger for glutamate/cystine, it is possible to use either radiolabeled glutamate or cystine as the substrate to study the function of this transporter. Since cystine can be taken up by multiple transporters, whereas the Na⁺-independent uptake of glutamate represents the function of system x_c⁻ exclusively [4], we chose to use [³H] glutamate as the substrate for uptake experiments. Cells, which were maintained in 75 cm² culture flasks in DMEM/F12 medium, were seeded in 24-well culture plates at an initial density of 0.1×10⁶ cells/well. In some experiments the inhibition of uptake by various competitors (unlabeled glutamate, cystine, cysteine, arginine, alanine, leucine, methionine

and phenylalanine) was examined to verify the presence of a functional system x_c^- in RGC-5 cells. Additional pharmacologic experiments using a range of concentrations [0.1 – 1000 μ M] of the known substrate for system x_c^- , quisqualate [29], were carried out to further establish the function of the transporter in RGC-5 cells. In remaining experiments, the effects of various NO donors on system x_c^- function were examined. For these experiments cells were treated on the second day following seeding with various NO donors: 3-nitroso-N-acetylpenicillamine (SNAP), S-nitrosoglutathione (SNOG), or 3-morpholiniosydnonimine hydrochloride (SIN-1) at concentrations of 10 μ M, 50 μ M, 100 μ M and 500 μ M, for 4, 6, 18 or 24 hours. Additional experiments were carried out using three other donors of reactive oxygen species (ROS), since oxidative stress has been shown to upregulate system x_c^- in other systems [4-9]. These ROS were menadione sodium bisulfite [5.4 μ M], hydrogen peroxide prepared from a 30% (W/W) solution (final concentration 0.0001%), and xanthine sodium salt/xanthine oxidase (concentrations 10 μ M and 2 mU/ml, respectively); the treatment time was 6 h. These concentrations were selected based on earlier reports of toxicity to retinal cells [30]. For uptake experiments, the culture medium was removed from the cells and they were washed twice with uptake buffer, which lacked Na^+ . The composition of the uptake buffer was 25 mM 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES)/Tris, 140 mM *N*-methyl-D-glucamine chloride, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , and 5 mM glucose, pH 7.5. Uptake was initiated by adding 250 μ l of uptake buffer containing [^3H]-glutamate. The cells were incubated for 15 min at 37°C, the medium was removed and the cells were washed thrice with ice-cold uptake buffer. The cells were solubilized with 0.5 ml of 1% sodium dodecyl sulfate-0.2N NaOH (SDS/NaOH) and used for determination of radioactivity by liquid scintillation spectrometry (Beckman LS 1701 scintillation counter, Beckman Instruments, Schaumburg, IL). This protocol is the same as described previously from our laboratory for the measurement of system x_c^- activity in RPE cells [4,16]. Saturation kinetics were analyzed by fitting the data to Michaelis-Menten equation. The Michaelis constant (K_m) was calculated by nonlinear regression analysis and then confirmed by linear regression. Experiments were repeated three times, and the results are expressed as the mean $\pm$ SE.

Semiquantitative RT-PCR analysis of xCT and 4F2hc mRNA

Initial studies were performed to establish the expression of xCT mRNA in RGC-5 cells compared with expression in mouse RPE cells, a positive control for xCT [4]. Subsequent analyses assessed xCT and 4F2hc in control cells and in cells treated with NO donors. Confluent RGC-5 cells were cultured in medium in the presence or absence of 50 μ M SNAP for 6 h. Total RNA was prepared using the TRIzol reagent. RT-PCR was carried out using primer pairs specific for xCT and 4F2hc. The primers for xCT were 5'-CCCAAGGGCATACTCCAGAAC-3' (sense) and 5'-CTCCGCACTGATGGTGGTAAA-3' (antisense) corresponding to nucleotide position 515-535 and 1184-1204, respectively, of mouse xCT cDNA [31]. The expected size of the PCR product, predicted from the positions of the primers is 690 bp. We used the mouse nucleotide sequence to design the xCT-specific RT-PCR primers for use with RGC-5 cells. The primers for 4F2hc were 5'-ATCCAAGGAGGAGCTATTCAAG-3' (sense) and 5'-CGTCCACTCAGCCAAGTAG-3' (antisense) corresponding to nucleotide position 367-389 and 949-971, respectively, of the rat 4F2hc cDNA. 18S RNA was used as the internal standard. RT-PCR was done at 30 cycles, with a denaturing phase of 1 min at 94°C, annealing phase of 1 min at 60°C, and an extension of 2 min at 72°C. 25 μ l of the PCR products were gel electrophoresed and stained with ethidium bromide.

Preparation of the antibody against xCT

There is no antibody commercially available for xCT. For generation of the antibody against xCT, we used the Antheprot Software program and determined that the peptide sequence

MVRKPVVATISKGGY, which corresponds to residues 1-15 of mouse xCT protein (Accession No. BAA90522), should be highly antigenic. An extra cysteine residue was added at the C-terminus of the peptide to permit conjugation with keyhole limpet hemocyanin. The peptide (2 mg) was conjugated to 2 mg of Imject maleimide activated mariculture keyhole limpet hemocyanin and purified by overnight dialysis. Approximately 300 µg of the peptide-conjugated hemocyanin in Freund's complete adjuvant was administered intradermally to New Zealand white rabbits (approximately 50 µl each). The initial injection was followed by two boosters. Antiserum was obtained 10 days after the second booster and purified using affinity chromatography.

Western blot analysis of xCT and 4F2hc

Initial studies were performed to detect xCT in RGC-5 and primary mouse ganglion cells and subsequent analyses were performed to detect both xCT and 4F2hc in RGC-5 cells treated with NO donors. For the latter experiments, RGC-5 cells were grown to confluence in DMEM:F12 medium and were incubated in the presence or absence of 50 µM SNAP for 6 h. In all cases to prepare protein, the medium was discarded and the cells were washed with 0.01 M PBS (pH 7.4) and lysed with the addition of cold lysis buffer (20 mM Tris-HCl, pH 7.5, containing 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate sodium, 0.1% SDS, 50 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSF). The sample was sonicated for 10-15 s and then unsolubilized debris was removed by centrifugation at 10,000 g for 10 min at 4°C. Protein concentration in the supernatant was determined using the Bio-rad assay reagent according to the manufacturer's instructions. Equivalent amounts of protein (10 or 30 µg) from the total cell lysates were boiled in Laemmli's buffer for 5 min and analyzed by 10% SDS-PAGE. After transferring the separated proteins onto nitrocellulose membranes, the membranes were blocked for 1.5 h at room temperature with Tris-buffered-saline-0.05% Tween-20 containing 5% non-fat milk. The membranes were incubated with rabbit affinity purified polyclonal anti-xCT antibody (1:1000) and goat polyclonal anti-4F2hc antibody (1:500) overnight at 4°C. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit and goat IgG antibody as the secondary antibody for 1.5 h, washed and the proteins visualized using ECL western blot detection system. The membranes were washed three times and blocked with 5% non-fat milk for 2 h. The membranes were re probed with mouse monoclonal anti-β-actin antibody (1:5000). Following immunoblotting, band density was determined using the imaging histogram feature of Adobe Photoshop 6.0. A drag-and-drop rectangular grid of identical size was placed on the band of interest and captured for processing. Background density was subtracted. Data represent an average of all the pixel values in the grid.

Immunocytochemical analysis of xCT and 4F2hc

RGC-5 cells or primary mouse ganglion cells were seeded onto coverslips and grown in either DMEM:F12 medium or the Neurobasal medium, respectively. The medium was removed; cells dried for 5 min, fixed in methanol for 5 min, and again air dried 5 min. They were washed three times in 0.01 M PBS (5 min). Samples were blocked with Power Block for 10 min and were incubated at room temperature for 3 h with anti-rabbit polyclonal anti-xCT antibody (1:500) and goat polyclonal anti-4F2hc antibody (1:100). Cells were washed three times with PBS (5 min) and were incubated with Cy-3-conjugated anti-rabbit IgG for xCT and Alexa Fluor 488-conjugated anti-goat IgG for 4F2hc. Negative control experiments were performed by blocking the anti-xCT antibody with 20 µg/ml of the antigenic peptide for 30 min before incubation with slides. In the case of 4F2hc, negative control experiments were performed by incubating the slides without the primary antibody. Samples were washed again with PBS and were incubated at room temperature with Hoescht dye 33342. After rinsing, slides were coverslipped with Vectashield mounting medium. The localization of the two proteins was determined by epifluorescence using a Zeiss Axioplan-2 microscope

equipped with an apotome, the axiovision program and an HRM camera. Intact eyes of mice, which had been embedded in OCT and prepared for cryosectioning, were processed for immunohistochemistry following our previously published protocol [16, 22] and retinas were examined using the Axiovision imaging system.

Glutathione Detection

Cellular levels of total GSH were determined using the Chemicon Glutathione Detection Kit. RGC-5 cells were grown in DMEM:F12 medium to confluency and incubated in the presence or absence of 50 μ M SNAP for 6 h. The medium was discarded, cells were washed twice with cold wash buffer and were incubated 10 min with lysis buffer.

Monochlorobimane (MCB) solution (supplied as part of the kit) was mixed with the cell lysate and incubated for 1 h at room temperature. The absorbance was measured using the 360/465 filter set of a Techan Ultra spectrophotometer (Research Park Triangle, NC). The standard curve for glutathione was established at concentrations 1 μ M - 10 μ M. The protein concentration of the lysate was assayed using the Bio-rad kit.

Data analysis

Each uptake experiment was performed in duplicate or triplicate and was repeated two to four times. Data analysis (analysis of variance) was performed using the SPSS statistical software package ($p < 0.05$ was considered significant). For data in which variances were equivalent the LSD post-hoc test was used and for tests in which they were not equivalent Dunnett's test was used. Kinetic analysis was done using Fig.P (version 6.0, Biosoft, Cambridge, UK). Data are presented as means $\pm$ SE.

Results

Molecular and functional evidence for the expression of system x_c^- in RGC-5 cells and in primary ganglion cells isolated from mouse retina

Earlier immunolocalization studies of intact retinal tissue suggested that system x_c^- was present in several retinal cell layers including the ganglion cell layer [16]. The presence of system x_c^- in the cultured RGC-5 cell line has not been reported. To provide evidence for the expression of system x_c^- in RGC-5 cells, RT-PCR was used to determine whether mRNA encoding xCT, the unique component of system x_c^- , was present in RGC-5 cells. Our earlier experiments demonstrated xCT expression in RPE, therefore we used RNA from mouse RPE [4] as a positive control for these studies. In the procedure for isolating mouse RPE, the neural retina is also isolated and hence we examined xCT expression in this tissue also. Fig. 1A shows detection by RT-PCR of the xCT component of system x_c^- in mouse RPE and neural retina as well as the rat RGC-5 cells. A 690 bp band was amplified in the presence of reverse transcriptase. The reaction gave no product if reverse transcriptase was not present. To establish whether the xCT protein was present in RGC-5 cells, Western blot analysis was performed. Extraction of protein from RGC-5 cells and subsequent SDS-PAGE and immunoblotting with our affinity purified antibody against xCT detected only one band with the expected molecular weight of ~50 kD (Fig. 1B). Additional evidence that the two components of system x_c^- , xCT and 4F2hc, colocalized was obtained by subjecting RGC-5 cells to immunocytochemical analysis using antibodies specific for these proteins (Fig. 1C). The first panel in Fig. 1C provides data for detection of xCT in RGC-5 cells and the second panel to detection of 4F2hc. In the merged image (third panel of Fig 1C), there are regions of co-localization in the RGC-5 cells.

To obtain functional evidence for the presence of system x_c^- in RGC-5 cells, the uptake of glutamate into these cells was studied in a Na^+ -free medium and the substrate specificity of the uptake process assessed by competition experiments. The cells took up [3H] glutamate in

the absence of Na^+ , suggesting the presence of Na^+ -independent transport mechanism(s) for glutamate in these cells. Competition experiments revealed that the Na^+ -independent uptake of [^3H] glutamate was inhibitable by glutamate, cystine and to a lesser degree by cysteine, but not by other amino acids such as arginine, alanine, serine, leucine, methionine or phenylalanine (Fig. 2A). The substrate specificity of the transport system mediating the Na^+ -independent uptake of glutamate in these cells matches with the known substrate specificity of system x_c^- . Additional pharmacologic experiments were conducted with quisqualic acid (quisqualate), a known substrate for system x_c^- [29]. In these studies increasing concentrations of quisqualate, which had been dissolved in the uptake buffer at pH 7.5, caused a dose-dependent inhibition of the uptake of [^3H] glutamate. The IC_{50} value (value at which 50% of the uptake of 3H glutamate was inhibited) for these studies was $3.71 \mu\text{M} \pm 0.28$.

Taken collectively, the data shown in Figs. 1 and 2 provide strong molecular and functional evidence that system x_c^- is present in RGC-5 cells. It has been demonstrated in some studies that when transformed cells are subjected to culture conditions, as is the case for the RGC-5 cells, they may express proteins that do not necessarily represent protein expression of the cells *in vivo*. An earlier report from our lab [16] showed a small photomicrograph of mouse retina and positive immunodetection of xCT and 4f2hc in the ganglion cell layer. In the present study, we repeated this experiment and examined cryosections of retinas incubated with the antibody against xCT (and 4f2hc, not shown) at a higher microscopic magnification. As shown in Fig. 3A, there is abundant immunopositive detection of xCT surrounding the cells of the ganglion cell layer as well as positive labeling of the radial glial fibers, representing the Müller cells. Since astrocytes and Müller cells can wrap their processes around ganglion cells, it can not be ruled out that the immunolabeling is of glial/astrocytic origin, rather than of the ganglion cell membranes themselves. To investigate this in a purified ganglion cell system, we used well-established immunopanning methods [28] and isolated ganglion cells from a neonatal mouse retinas. Fig. 3B shows a differential interference contrast micrograph of primary ganglion cells that had been grown for three days in the culture medium described above. Note the long processes and the variably sized cell bodies characteristic of primary ganglion cells. Fig. 3C shows immunolabeling of the primary cells with an antibody for Thy-1, considered a marker for ganglion cells, and companion experiments immunolabeling for xCT. The cells were positive also for the 4F2hc component of system x_c^- (data not shown). Fig. 3D shows immunoblotting for detection of xCT in the purified primary mouse ganglion cells isolated from two separate protein preparations. In these studies, the isolated by immunopanning and proteins prepared immediately (with no cell culture to ensure a large population of cells and a good protein yield). Immunoblotting studies detected the expected band of ~50 kD for xCT in two separate preparations of proteins. β -actin was used as a positive control for the immunoblotting experiments. These data obtained in the intact mouse retinal tissue and primary ganglion cells provide strong support for the presence of system x_c^- . Because the primary neurons do not proliferate, it is not feasible to study the functional aspects of system x_c^- in the primary cell culture, thus the remainder of the experiments were performed with the RGC-5 cells.

Influence of donors of NO and ROS on the function of system x_c^- in RGC-5 cells

Treatment of confluent cultures of RGC-5 cells with the NO donors SNAP, SNOG or SIN-1 for 4 h showed no change in the uptake of [^3H] glutamate compared with control or vehicle (0.05% DMSO) cells (data not shown). After 6 h treatment, however, incubation of RGC-5 cells with NO donors resulted in significant stimulation of glutamate uptake. Specifically, incubation of RGC-5 cells with SNAP at concentrations of 50 and 100 μM led to ~80% increase in system x_c^- activity (Fig.4A). Incubations with high (500 μM) concentrations of

SNAP resulted in decreased activity of system x_c^- and may reflect toxic effects on the cells. Treatment of RGC-5 cells for 6 h with the NO donor compound SNOG at concentrations of 50 and 100 μM led to $\sim 20\%$ increase in system x_c^- activity (Fig. 4B), while exposure of cells to SIN-1 did not influence the activity of system x_c^- (Fig. 4C). Incubation of RGC-5 cells with SNAP and SNOG at 50 μM concentration for 18 h led to a similar stimulation of system x_c^- activity (Fig. 5A and 5B) as observed with 6 h. Interestingly, 24 h incubation of RGC-5 cells no longer showed stimulation of system x_c^- activity (data not shown). All effects observed with the NO donors used freshly-prepared solutions; incubation of cells with “spent” SNAP or SNOG showed a markedly reduced stimulatory effect (data not shown). To confirm the sensitivity of system x_c^- in RGC-5 cells to other forms of oxidative stress, cells were treated with donors of reactive oxygen species (ROS) xanthine/xanthine oxidase, menadione sodium bisulfite or hydrogen peroxide. Treatment with xanthine/xanthine oxidase, a donor of extracellular ROS, led to $\sim 45\%$ increase in system x_c^- activity, while incubation with menadione sodium bisulfite, a donor of intracellular ROS had no effect on the activity of system x_c^- (Fig. 6). Hydrogen peroxide stimulated the activity of the transporter in RGC-5 cells by $\sim 20\%$. To characterize the effects of oxidative stress on the kinetics and regulation of system x_c^- in RGC-5 cells, subsequent experiments used SNAP at a concentration of 50 μM for 6 h.

To determine whether the increased activity induced by SNAP on system x_c^- activity was a generalized effect on all transporter proteins in RGC-5 cells, or whether it was specific, RGC-5 cells were incubated in the presence or absence of 50 μM SNAP and the effect on the uptake of radiolabeled amino acids, alanine and leucine was compared to that of glutamate. As shown in Fig. 7, incubation of cells with 50 μM SNAP stimulated the Na^+ -independent uptake of glutamate as demonstrated previously (Figs. 4 and 5); exposure of cells to 50 μM SNAP had no effect however on the Na^+ -independent uptake of alanine or leucine.

Kinetic analysis of system x_c^- in RGC-5 cells exposed to the NO donor SNAP

The kinetics of system x_c^- activity in RGC-5 cells was analyzed in control cells and in cells treated with SNAP (Fig. 8). The transport function of system x_c^- was monitored by measuring the Na^+ -independent uptake of glutamate and the analysis showed that the increase in the transport activity of system x_c^- observed in SNAP-treated RGC-5 cells compared to control cells was associated primarily with an increase in the maximal velocity of the transporter with no significant change in the substrate affinity. The maximal velocity of glutamate uptake was 1.6-fold greater in SNAP-treated cells than in control cells (13.3 ± 0.5 vs. 9.5 ± 0.5 nmol/mg of protein/15 min). The Michaelis constant for glutamate in SNAP-treated cells was similar to control cells (0.11 ± 0.02 versus 0.12 ± 0.02 mM).

Regulation of the expression of system x_c^- by NO in RGC-5 cells

To determine whether system x_c^- expression was regulated by NO, we studied the influence of SNAP treatment on the steady-state levels of mRNA transcripts specific for 4F2hc and xCT, the two protein components of system x_c^- . mRNA samples isolated from control and SNAP-treated RGC-5 cells were used for semiquantitative RT-PCR for the determination of the levels of mRNA transcripts. As an internal control, the steady-state levels of the 18S RNA were determined in the mRNA samples in parallel. Data were expressed as the ratio of the band density of the gene of interest compared to that of 18S RNA in the same sample. The results showed that treatment with SNAP did not alter the steady-state levels of 4F2hc mRNA (Fig. 8). In contrast, the steady-state levels of xCT mRNA increased markedly (by $\sim 50\%$) in SNAP-treated cells compared to control cells. These results suggest that the SNAP-induced increase in the transport activity of system x_c^- is associated with an increase in the expression of xCT with no significant change in the expression of 4F2hc. To

determine whether the levels of 4F2hc and xCT were similarly altered, proteins were extracted from RGC-5 cells incubated in the presence or absence of SNAP and used for Western analysis. β -actin was used as an internal control. Immunoblotting with antibodies against 4F2hc showed no difference between the SNAP-treated cells versus control cells, however, levels of xCT were significantly greater in the SNAP-treated cells than in control cells (Fig. 10).

Glutathione levels in RGC-5 cells treated with the NO donor SNAP

Given that system x_c^- is an exchanger for glutamate and cystine facilitating the synthesis of the antioxidant glutathione, coupled with the finding that the NO donor SNAP increases the activity of system x_c^- , we asked whether glutathione levels were altered when RGC-5 cells were treated with SNAP. Control cells, which were incubated in medium without SNAP or in 0.05% DMSO-containing medium (vehicle control) had GSH levels of 1.87 ± 0.07 and 1.73 ± 0.13 nmol/mg of protein, respectively. Cells treated with 50 or 100 μ M SNAP had GSH levels of 2.94 ± 0.25 and 2.98 ± 0.17 nmol/mg of protein, respectively. These data suggest that system x_c^- may contribute to generation of GSH providing protection during oxidative stress to RGC-5 cells.

Discussion

The present study examined system x_c^- in retinal ganglion cells. Earlier immunohistochemical studies from our laboratory [13] had detected system x_c^- in the ganglion cell layer of the intact mouse retina. In the present study, we repeated this experiment to confirm at a higher power magnification whether our affinity purified antibody against xCT detected the protein in ganglion cells. The immunolabeling was abundant around the cell bodies of cells in the ganglion cell layer and was positive also in the radial fibers of the Müller cells. Since Müller are known to ensheath the ganglion cells, which could account for the immunolabeling, it was necessary to purify ganglion cells from the mouse retina and perform immunocytochemical studies and immunoblotting on the purified ganglion cell population. Both the immunocytochemical studies and the immunoblotting of the protein isolated from the purified ganglion cells detected robust levels of the xCT protein as well as the 4F2hc protein, the other component of system x_c^- . In our studies of primary ganglion cells we followed the well established methods of Barres [28] to isolate the cells and used the marker Thy-1 to verify that our cells were positive for this marker. The pattern of growth of the cells in culture further suggested that indeed we were studying isolated ganglion cells. Unfortunately, the primary ganglion cells do not proliferate in culture, while this provides further evidence of their purity; it severely limits their usefulness in functional studies that require a confluent population of cells.

To understand how system x_c^- functions in cells requires a method whereby sufficient numbers of cells can be cultured and their uptake of radiolabeled substrates examined. We used the rat ganglion cell line RGC-5 to test the function of system x_c^- . This cell line expresses markers of ganglion cells, but not markers for other retinal cells types, such as Müller cells and has been described as a promising tool for studies of ganglion cell function [18, 27]. We used molecular, biochemical, immunohistochemical and functional methods to establish that system x_c^- was present in these cells prior to studying its regulation. RT-PCR, cloning and sequencing data established the molecular identity of xCT gene in RGC-5 cells. Our affinity purified antibody prepared against a highly antigenic peptide sequence of xCT detected the protein with the appropriate molecular weight. Similarly, 4F2hc, which functions with xCT in system x_c^- , was detected in protein extracts of RGC-5 cells. Our immunohistochemical studies co-localized the two proteins in the cultured cells. Functional studies using various unlabeled anion acids to inhibit the Na^+ -independent uptake of radiolabeled glutamate clearly demonstrated inhibition by the known substrates of system

x_c^- . Additional pharmacological studies using quisqualate, a substrate specific for system x_c^- [29], also showed increasing inhibition of glutamate uptake as levels of quisqualate were increased.

Having demonstrated the presence of system x_c^- in RGC-5 cells, we asked whether nitric oxide (NO) would regulate the expression and activity of the transporter. Our data show clearly that SNAP and SNOG, two donors of NO, increase the activity of system x_c^- , when exposed to the cells for as few as 6 h. The increased activity was associated with an increase in the maximum velocity of the transporter. The molecular analysis of the two components of system x_c^- showed that there was increased expression of the mRNA encoding xCT, with no change in expression of 4F2hc. Similar results were observed when the quantity of the two proteins was determined in the cells treated with SNAP versus controls. That is, the level of xCT increased, while the level of 4F2hc remained unchanged. Following exposure to NO donors, the level of glutathione increased in RGC-5 cells, which is anticipated since system x_c^- transports constituents of this antioxidant. In addition to the effects of NO donors on system x_c^- , we asked whether other agents, known to induce oxidative stress, such as donors of reactive oxygen species and hydrogen peroxide would also stimulate the activity of system x_c^- and functional assays showed that they did.

The effects of NO on system x_c^- were of interest because NO is thought to play a role in diabetic retinopathy. NO levels are increased in diabetes, including in patients with diabetic retinopathy [23,24]. NO is a key factor leading to neuronal death triggered by glutamate-induced stimulation of the NMDA receptor. This has been demonstrated in many neuronal cell types [32-34] including retinal neurons [33,35]. In retina, a low concentration of NO plays a protective role in glutamate neurotoxicity by closing the NMDA receptor gated ion channel, but elevated levels of NO, interacting with oxygen radicals, become toxic and mediate glutamate-induced neurotoxicity [35,36].

The finding that NO stimulates the activity of system x_c^- raises an important question, that of whether the long-term consequences of increased activity of this transporter are beneficial or could be potentially deleterious. This issue has been raised in studies of system x_c^- in brain slices [36]. While a system that provides necessary constituents for synthesis of glutathione would arguably be beneficial, it must be kept in mind also that when system x_c^- is functioning *in vivo*, it is exchanging glutamate for cystine. That is cystine is being taken up by cells, but glutamate is being released into the extracellular milieu. Given that glutamate is toxic to ganglion cells via stimulation of the NMDA receptor [37], this could be a deleterious consequence of excess activity of system x_c^- . It would be imperative that in any retinal condition in which system x_c^- is upregulated that retinal Müller cells be capable of taking up excess glutamate and converting it to glutamine. Interestingly, our data show that while 6 and 18 h incubation with NO donors led to increased activity of system x_c^- , the activity returned to baseline by 24 h exposure to NO donors, which may suggest a self-regulating system in these cells. Compromised function of Müller cells during diabetes, including decreased activity of glutamate transporters which has been reported by some investigators [38], could have a major impact on whether upregulation of system x_c^- in diabetes would be entirely beneficial. It remains to be tested whether system x_c^- in ganglion cells isolated from an *in vivo* model of diabetes (such as rat or mouse) is upregulated as our *in vitro* data using NO would suggest. Future studies will address the function of system x_c^- in ganglion cells isolated from diabetic models.

It has been reported that in retina, GSH is localized primarily to Müller cells and horizontal cells [39]. This study did however describe low level GSH in other retinal neurons. It is recognized that system x_c^- has been detected more frequently in glial cells than other cell types [2], which has led to a model whereby system x_c^- is thought to be primarily a

transporter active in glial cells. More recent studies detecting system x_c^- in neurons [10,12] requires that the model be revisited. Allen et al [10] point out that while a number of experiments present data suggesting that neurons rely exclusively on GSH effluxed from astrocytes [40,41], other studies have shown that cultured neurons (such as hippocampal neurons) readily accumulate cystine. These results agree with findings by others demonstrating that primary cultures of either cortical or hippocampal neurons accumulate ^{35}S -cystine [42,43]. There is no disputing that astrocytes play a key role in providing GSH for neurons, but it seems that the transporter for cystine, system x_c^- is present in at least some primary neuronal cell cultures and may be functional. Our data provide strong evidence for the presence of the transporter system in primary ganglion cells of the retina. Our findings that the ganglion cell line, RGC-5 upregulates this system under situations of oxidative stress that can occur in retinal disease, may be relevant to diseases such as diabetic retinopathy.

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Fig. 1. Expression of xCT in RGC-5 cells and evidence for functional activity of system x_c^-
 (A) RT-PCR analysis of xCT-specific mRNA transcript in mouse RPE and the RGC-5 cell line. Total RNA was isolated from mouse RPE, isolated mouse neural retina and rat ganglion cells (RGC-5), RT-PCR was carried out with primers specific for mouse or rat xCT mRNA. As negative controls, the samples were subjected to RT-PCR without reverse transcriptase (-RT). DNA standards (75 bp–12.2 kbp) were run on the gel in parallel to determine the size of the RT-PCR products. (B) Immunoblotting of membranes containing protein isolated from two different preparations of RGC-5 cells using an affinity purified antibody against xCT. Both lanes represent 10 μ g protein loaded onto the gel. (C) Immunodetection of the two components of system x_c^- , xCT and 4F2hc. In the first panel (labeled xCT), the protein was detected using our affinity purified antibody and a Cy-3-labeled secondary antibody (red), the nuclei fluoresce blue due to incubation of the slides with Hoescht dye 33342; in the second panel (labeled 4F2hc), the protein was detected using anti-4F2hc antibody followed by an Alexa 488-conjugated secondary antibody (green); the third panel is a merged image using 3 filters to detect Cy-3, Alexa 488 and Hoescht 33342. Areas where xCT and 4F2hc are co-localized have an orange-yellow appearance due to overlap of the green and red signals.

Fig. 2. Functional evidence for system x_c^- in RGC-5 cells

(A) Inhibition of Na^+ -independent uptake of [³H] glutamate by various amino acids in RGC-5 cells. Uptake of [³H]glutamate (2.5 μM) was measured in confluent RGC-5 cells in Na^+ -free medium for 15 minutes at 37°C in the absence or presence of 2 mM unlabeled amino acids (except for cystine, 1.0 mM). Results are presented as percent of control uptake measured in the absence of unlabeled amino acids. The values for control uptake (100%) were 182.6 ± 3.42 pmol/mg protein/15 minutes for [³H] glutamate. Values represent means $\pm$ SE for three determinations from two independent experiments. (Con, control; Glu, glutamate; Cyt, cystine; Cys, cysteine; Arg, arginine; Ala, alanine; Ser, serine; Leu, leucine; Met, methionine; Phe, phenylalanine). (Significantly different from control, *** $p < 0.001$).

(B) Dose-dependent inhibition of the Na⁺-independent uptake of [³H] glutamate with increasing concentrations of quisqualic acid. Cells were cultured as described in the text and at the time of the experiment they were incubated in quisqualate which had been dissolved in the uptake buffer over a concentration range of 0.1 – 1000 μM. Values represent means ± SE for three determinations from three independent experiments.

Fig. 3. Detection of components of system x_c^- in retinal ganglion cells in vivo

(A) Cryosection (10 μm thick) of retina of adult mouse that had been incubated overnight with the affinity purified antibody against xCT (1:500) followed by incubation with the secondary antibody cy-3 (1:1000) (Magnification = 400X). The cells of the ganglion cell layer (gcl) show abundant positive immunolabeling surrounding each cell (arrowheads). The vertically oriented radial fibers reflect positive immunolabeling of xCT in the Müller cells. (B) DIC (differential interference contrast) image of primary ganglion cells isolated from neonatal mouse retinas that had been grown for 3 days in the medium described in the text. The cells have long processes extending from the variably sized cell bodies, both of which are characteristics of primary ganglion cells. (C) Immunolabeling of primary mouse

ganglion cells with Thy-1, a marker for these cells and immunobabeling with xCT. (Magnification = 630X). (D) Immunoblotting of xCT in primary mouse ganglion cells. The cells were purified by immunopanning and immediately subjected to protein isolation, SDS-Page and immunoblotting for xCT and for β -actin. Lanes 1 and 2 represent two separate preparations from purified ganglion cells.

Fig. 4. Stimulation of the Na^+ -independent uptake of glutamate in RGC-5 cells following 6 h incubation with NO donors

Confluent RGC-5 cells were incubated at 37°C for 6 h in regular DMEM:F12 medium (control), DMEM:F12 containing 0.05% DMSO (vehicle control) or in medium containing the NO donors (dissolved in DMSO) (A) 3-Nitroso-N-acetylpenicillamine (SNAP), (B) S-Nitrosoglutathione (SNOG), or (C) 3-Morpholinosydnonimine hydrochloride (SIN-1) at concentrations of 10 μM , 50 μM , 100 μM and 500 μM . Uptake of $[^3\text{H}]$ glutamate (2.5 μM) was measured in these cells for 15 minutes at 37°C in a Na^+ -free medium. Values are means $\pm$ SE for three determinations from two independent experiments. (Significantly different from DMSO vehicle control, *** $p < 0.001$, ** $p < 0.01$).

Fig. 5. Stimulation of the Na⁺-independent uptake of glutamate in RGC-5 cells following 18 h incubation with NO donors

Confluent RGC-5 cells were incubated at 37°C for 18 h in regular DMEM:F12 medium (control), DMEM:F12 containing 0.05% DMSO (vehicle control) or in medium containing the NO donors (dissolved in DMSO) (A) 3-Nitroso-N-acetylpenicillamine (SNAP), or (B) S-Nitrosoglutathione (SNOG) at concentrations of 10 μM, 50 μM, 100 μM and 500 μM. Uptake of [³H]glutamate (2.5 μM) was measured in these cells for 15 minutes at 37°C in a Na⁺-free medium. Values are means ± SE for three determinations from two independent experiments. (Significantly difference from DMSO vehicle control, ** p < 0.01, * p < 0.05)

Fig. 6. Stimulation of the Na⁺-independent uptake of glutamate in RGC-5 cells following 6 h incubation with donors of reactive oxygen species (ROS)

Confluent RGC-5 cells were incubated at 37°C for 6 h in regular DMEM:F12 medium or in medium containing the ROS donors xanthine sodium salt/xanthine oxidase [10 μM/2 mU/ml]; hydrogen peroxide [0.0001%], and menadione sodium bisulfite [5.4 μM]. Uptake of [³H]glutamate (2.5 μM) was measured in these cells for 15 minutes at 37°C in a Na⁺-free medium. Values are means ± SE for three determinations from two independent experiments. (Significantly different from control, ***p < 0.001)

Fig. 7. Substrate specificity of the NO-induced stimulation of system x_c^- activity

Confluent RGC-5 cells were incubated at 37°C for 6 h in the presence or absence of 50 μ M SNAP and the effect on the uptake of [3 H] glutamate (2.5 μ M), [3 H] alanine (2.5 μ M), or [3 H] leucine (2.5 μ M) was measured in these cells for 15 minutes at 37°C in a Na^+ -free medium. Values are means $\pm$ SE for three determinations from two independent experiments. (Significantly different from control, *** $p < 0.001$.)

Fig. 8. Kinetic analysis of system x_c^- in control and SNAP-treated RGC-5 cells
RGC-5 cells were treated 6 h in the absence or presence of 50 μ M SNAP following which the uptake of glutamate was measured in a Na^+ -free medium for 15 min at 37°C over a glutamate concentration range of 0.05 to 1.0 mM. Values are means $\pm$ SE for six determinations from two separate experiments. *Inset*: Eadie–Hofstee plot.

Figure 9. Effect of SNAP on the steady-state levels of mRNA for xCT and 4F2hc in RGC-5 cells Confluent RGC-5 cells were incubated in the absence or presence of 50 μ M SNAP for 6 h at 37°C. Total RNA was isolated and used for semiquantitative RT-PCR. Primer pairs specific for rat xCT mRNA (690 bp) and rat 4F2hc (605 bp) were used. 18S RNA (315 bp) was analyzed in the same RNA samples as the internal control. RT-PCR was done with a wide range of PCR cycles (9–30). The resultant products were run on a gel and stained with ethidium bromide. Intensity of the band was quantified as described in the methods. *A*: a representative ethidium bromide gel. *B*: band intensity of cDNA (4F2hc or xCT) of interest relative to 18S cDNA band intensity in control and SNAP-treated cells. DNA standards (100 bp~12.2 kbp) were run on the gel in parallel to determine the size of the RT-PCR product. (M, DNA marker; C, control; S, SNAP-treated cells, ** $p < 0.01$)

Figure 10. Effect of SNAP on the levels of xCT and 4F2hc proteins in RGC-5 cells

Confluent RGC-5 cells were incubated in the absence or presence of 50 μ M SNAP for 6 h at 37°C. Proteins were extracted from cells and subjected to SDS-PAGE, followed by immunoblotting using an affinity purified antibody against 4F2hc, M_r ~90 (A) or against xCT, M_r ~50 (C) as well as an antibody against β -actin, M_r ~45 (internal loading control). The density of the bands was quantified by densitometry. Data from densitometric scans of blots: (B) 4F2hc/ β actin and (D) xCT/ β -actin. The 4F2hc/ β -actin band or the xCT/ β -actin ratio in control cells was taken as 1, ** $p < 0.01$)