Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 326 (2005) 371–377
BBRC
www.elsevier.com/locate/ybbrc

Coordinate regulation of glutathione metabolism in astrocytes by Nrf2

Xiaojian Sun[a], Heidi Erb[a], Timothy H. Murphy[a,b,*]

[a] *Department of Psychiatry, Kinsmen Laboratory of Neurological Research and Brain Research Center, 4835-2255 Wesbrook Mall, University of British Columbia, Vancouver, BC, Canada V6T 1Z3*
[b] *Department of Physiology, Kinsmen Laboratory of Neurological Research and Brain Research Center, 4835-2255 Wesbrook Mall, University of British Columbia, Vancouver, BC, Canada V6T 1Z3*

Received 8 November 2004
Available online 18 November 2004

Abstract

The tripeptide glutathione (GSH) represents the major brain thiol and is essential for prevention of oxidative stress. Using monochlorobimane to label intracellular GSH in a glutathione *S*-transferase catalyzed reaction we have examined the kinetics of GSH metabolism including its rate of conjugation, total GSH content, synthesis, and efflux in astrocyte cultures under basal conditions and after induction of antioxidant response element (ARE)-mediated gene expression by the transcription factor Nrf2. In the presence of a cerebral spinal fluid-like salt solution astrocytes could not synthesize detectable levels of GSH. Addition of GSH precursors, cystine, glutamate, and glycine, rapidly restored GSH synthesis. Astrocytes were able to use either glutamate or glutamine as precursors equally for GSH synthesis. Using the small molecule chemical inducer *tert*-butylhydroqunione (tBHQ) we report that induction of ARE-mediated gene expression is associated with a coordinated increase in GSH content and synthesis rate with little effect on the rate of GSH conjugation or efflux. Consistent with the effect of the inducer, adenovirus-mediated overexpression of the transcription factor Nrf2 that mediates tBHQ's effects also increased GSH content, confirming that GSH metabolism can be regulated by the Nrf2 pathway.

Keywords: Glutathione; Monochlorobimane; Phase II detoxification enzymes; Nrf2; *tert*-Butylhydroquinone; Antioxidant response element; Oxidative stress; Neuroprotection

The tripeptide glutathione (GSH; γ-L-glutamyl-L-cysteinyl-glycine) is the most abundant thiol in mammalian cells with concentrations of up to 12 mM [9]. It is a central component of the antioxidant defences of cells, acting both to directly detoxify reactive oxygen species (ROS) and as a cofactor for several peroxidases [11]. It can also detoxify xenobiotics by forming S-conjugates, catalyzed by glutathione *S*-transferase (GST). A compromised GSH system in the brain has been connected with oxidative stress occurring in neurological diseases, such as Parkinson's disease (PD), Alzheimer's disease, Huntington's disease, and stroke [14]. Examining the mechanism of glutathione metabolism will provide insight into the understanding of how brain copes with oxidative stress and may provide therapeutic insight.

It is well established that, in the central nervous system, there is a compartmentalization of GSH between neurons and glia, with glia, especially astrocytes, in general exhibiting higher levels of GSH [29,30]. The function of this astrocytic GSH is unclear. Some studies suggest that it may be exported to neighboring cells [36] to provide antioxidant substrates. Cell culture is an attractive model for examining the function of astrocytic GSH since relatively pure populations of astrocytes can be cultivated [34].

Since GSH plays a pivotal role in protecting cells, there has been a considerable interest in the development of methods to quantify the cellular GSH content.

* Corresponding author. Fax: +1 604 822 7981.
E-mail address: thmurphy@interchange.ubc.ca (T.H. Murphy).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.11.031

There are mainly two common methods for determining the GSH level in cells. One is using reversed-phase high-performance liquid chromatography (HPLC) following derivatization with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), which is reliable and sensitive but time consuming and laborious [23]. The other commonly used technique is by adding monochlorobimane (MCB) to cell culture medium and allowing intracellular glutathione *S*-transferases to form GSH–MCB conjugates that have a high fluorescence and can be measured by flow cytometry [16]. The latter one can be used to measure GSH level in living cells and is simpler to perform, while giving the identical results with the first method [22]. Recently, it was established that MCB was a specific probe for measuring the intracellular GSH level in astrocyte cultures, its conjugation with GSH being enzymatically catalyzed by GST isoenzymes to form the highly fluorescent conjugate [7]. Although these methods provide information about GSH content they do not necessarily provide information about other parameters associated with its synthesis and metabolism.

In the present study, we monitor the metabolism of GSH in astrocytes, using a fluorescence plate reader and monitoring the kinetics of the GST catalyzed reaction between GSH and the substrate monochlorobimane (MCB). We were able to measure various kinetic parameters of GSH metabolism, such as the rates of GSH synthesis, conjugation and efflux under basal conditions and after induction of Nrf2 driven antioxidant response element-mediated gene expression. This assay indicates coordinate regulation of GSH metabolism by Nrf2 and supports previous microarray data showing up-regulation of mRNAs for this pathway in astrocytes [32].

Materials and methods

Materials. Reduced glutathione, glutathione *S*-transferase, glutamine, cysteine, glutamic acid, *tert*-butylhydroquinone (tBHQ), L-buthionine-sulfoximine (BSO), glucose, Hepes, NaCl, KCl, and Na_2HPO_4 $NaHCO_3$ were purchased from Sigma–Aldrich. Monochlorobimane was purchased from either Fluka or Molecular Probes. MK-571 was purchased from Alexis Biochemicals.

Astrocyte culture. Astrocyte cultures were prepared from 0- to 2-day-old Wistar rats. The animals were anesthetized with cold, sacrificed by decapitation, the skulls were opened, and the brains were removed in sterile PBS. The meninges were carefully removed from the brains. The remaining cortices were digested in papain at 37 °C for 10 min, and the cells plated in MEM (Invitrogen Cat# 51200-038) were amended to 15.6 mM glucose, 2 mM glutamine, 10% fetal bovine serum, and 100 U/mL penicillin/streptomycin (Invitrogen Cat# 15140). Cells were passaged by trypsinization at 1 week at a concentration to yield 75–90% confluence on the day of the MCB assay. Cells were plated into 24-well plates from either Corning or Falcon. To inhibit the GSH synthesis, the cultures were incubated with 10 μM BSO overnight. To induce the phase II pathway, the cultures were incubated with 20 μM tBHQ overnight. For adenovirus infection, the cultures were infected at 1DIV by virus diluted to a multiplicity of infection of 200 in MEM-pyr. The cultures were allowed to express the transgenes for 48 h before usage. The recombinant adenoviruses were constructed in our laboratory previously [32].

Plate reader assay. Fluorometric assays were performed using a plate reader (Fluoroskan Ascent FL); $\lambda_{ex} = 355 \pm 19$ nm, $\lambda_{em} = 527 \pm 5$ nm. Note we chose these excitation/emission filters, which are sub-optimal for MCB detection since they prevented saturation of the detector within the plate reader permitting linearity between the concentration of MCB–GSH and detected fluorescence over the range associated with the astrocyte fluorescence values. MCB was added to the cells at 10 μM unless otherwise indicated. All cell reactions were carried out in Hanks' Balanced Salt Solution (HBSS) (138 mM NaCl, 5 mM KCl, 0.34 mM Na_2HPO_4, 10 mM Na^+ Hepes, 1 mM $NaHCO_3$, 20 mM glucose, 2.5 mM $CaCl_2$, and 1 mM $MgSO_4$). During the assay cells were bathed in either HBSS as a control or HBSS with the following: 100 μM GSH, 100 μM cystine, or a 100 μM cocktail of GSH precursors (glutamate, cystine, and glycine). Efflux was estimated by measuring the change in cell fluorescence after changing the media. To inhibit the efflux, 25 μM MK571 was added to the cultures. Fluorescence images were collected at Axiophot photomicroscope (Zeiss) [excitation (ex): 340–380 nm, emission (em): 435–485 nm].

Data analysis. All experiments were done at least five different times unless otherwise stated. Results are presented as means ± SE. Statistical analysis of raw data was performed with Excel and Origin. Experimental groups were compared by Student's *t* test. A statistical probability of $p < 0.05$ was considered significant.

Results and discussion

Specificity of monochlorobimane labelling

Monochlorobimane (MCB) has been proven to be the most specific probe for measuring intracellular glutathione (GSH) level in different mammalian cell types [8,35]. The formation of a highly fluorescent GSH–MCB conjugate is catalyzed by glutathione *S*-transferase (GST) which has three isoforms, alpha (α), mu (μ), and pi (π), with the μ form being the most efficient. The μGST isoform is present in neurons and glia, and in most brain regions [4]. Therefore, we assumed that MCB is also a specific probe for measuring GSH levels in astrocyte cultures. It has been established that after labelling cultured astrocytes with MCB, the majority of the fluorescence signal is in the acid-soluble fraction, rather than protein fraction, indicating a low binding to protein-SH groups [7]. We further tested the specificity of MCB by in vitro experiments using purified glutathione *S*-transferase, the reduced form GSH, and MCB to produce a fluorescent GSH–MCB adduct. In the absence of GST, addition of 200 μM GSH to 10 μM MCB resulted in a modest linear increase in fluorescent product over time. Addition of purified GST resulted in a robust exponential increase in the fluorescence intensity, indicating that this is an enzyme-catalyzed reaction, following first order kinetics. The reaction reached a plateau that was related to the amount of GSH and MCB added, while the conjugation rate is proportional to the GST amount. The time constants of the exponential phase are 1436.6, 690.6, and 343.5 s, respectively for 0.01, 0.02, and 0.04 U of GST.