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Research Paper

Effect of Nrf2 activators on release of glutathione, cysteinylglycine and homocysteine by human U373 astroglial cells ☆

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ABSTRACT

Neurons rely on the release and subsequent cleavage of GSH to cysteinylglycine (CysGly) by astrocytes in order to maintain optimal intracellular GSH levels. In neurodegenerative diseases characterised by oxidative stress, neurons need an optimal GSH supply to defend themselves against free radicals released from activated microglia and astroglia. The rate of GSH synthesis is controlled largely by the activity of γ-glutamyl cysteine ligase. Expression of γ-glutamyl cysteine ligase and of the Xc- system, which facilitates cystine uptake, is regulated by the redox-sensitive transcription factor, nuclear factor erythroid-2-related factor 2 (Nrf2). Compounds that can activate the Nrf2-ARE pathway, referred to as 'Nrf2 activators' are receiving growing attention due to their potential as GSH-boosting drugs.

This study compares four known Nrf2 activators, R-α-Lipoic acid (LA), tert-butylhydroquinone (TBHQ), sulforaphane (SFN) and *Polygonum cuspidatum* extract containing 50% resveratrol (PC-Res) for their effects on astroglial release of GSH and CysGly. GSH levels increased dose-dependently in response to all four drugs. Sulforaphane produced the most potent effect, increasing GSH by up to 2.4-fold. PC-Res increased GSH up to 1.6-fold, followed by TBHQ (1.5-fold) and LA (1.4-fold). GSH is processed by the ectoenzyme, γ-glutamyl transpeptidase, to form CysGly. Once again, SFN produced the most potent effect, increasing CysGly by up to 1.7-fold, compared to control cells. TBHQ and PC-Res both induced fold increases of 1.3, followed by LA with a fold increase of 1.2. The results from the present study showed that sulforaphane, followed by lipoic acid, resveratrol and *Polygonum multiflorum* were all identified as potent "GSH and Cys-Gly boosters".

Introduction

Oxidative stress, defined as an imbalance between the production and detoxification of reactive oxygen species (ROS), is thought to play a significant role in the neurodegeneration evident in Alzheimer's disease (AD) [15].

Glutathione (GSH), a tripeptide consisting of glutamate, cysteine and glycine is the key regulator of the intracellular redox state. It can non-enzymatically detoxify ROS, such as superoxide and hydroxyl radicals, as well as act as an electron donor for the reduction of peroxides, catalysed by glutathione peroxidase [4]. GSH is synthesised from its constituent amino acids by the sequential action of two enzymes, the rate-limiting enzyme γ-glutamyl cysteine ligase and glutathione synthetase [24].

In the brain, neurons rely on the release and subsequent cleavage of GSH by astrocytes in order to maintain optimal intracellular GSH levels [9]. Extracellular GSH released from astrocytes is metabolised by γ-glutamyl transpeptidase to form the dipeptide, cysteinylglycine (CysGly), which is then processed by the neuronal ectopeptidase, aminopeptidase N, allowing neurons to immediately take up the resultant cysteine and glycine.

In neurodegenerative diseases such as AD, neurons need an optimal GSH supply to defend themselves against free radicals, such as superoxide and nitric oxide, released from activated microglia and astrocytes [2,32]. Therapeutic strategies enabling astrocytes to provide neurons with sufficient substrates for GSH

Abbreviations: ARE, antioxidant response elements; CysGly, cysteinylglycine; DMEM, Dulbeccos's Modified Eagle Medium; GSH, glutathione; HCys, homocysteine; Nrf2, nuclear factor erythroid-2-related factor 2; LA, α-lipoic acid; PC, *Polygonum cuspidatum*; ROS, reactive oxygen species; SFN, sulforaphane; TBHQ, Tert-butylhydroquinone

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synthesis is of particular interest as reductions in neuronal GSH levels may contribute to neuronal cell death in a pro-oxidative, pro-inflammatory environment.

The rate of GSH synthesis is controlled largely by the activity of γ-glutamyl cysteine ligase, the first enzyme required for GSH synthesis, and by the availability of cysteine/cystine [10,12,3]. Expression of the catalytic and modulatory subunits of γ-glutamyl cysteine ligase and of the Xc-system, which facilitates cystine uptake, are regulated by the redox-sensitive transcription factor, nuclear factor erythroid-2-related factor 2 (Nrf2) [8]. Under basal conditions, Nrf2 interacts with Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm and undergoes ubiquitin-mediated proteasomal degradation [25]. Upon oxidative modification of cysteine residues within Keap1, Keap1 dissociates from Nrf2, permitting Nrf2 translocation into the nucleus. Once in the nucleus, Nrf2 binds to antioxidant response elements (ARE) present in the regulatory regions of a range of phase II antioxidant defence genes, including numerous GSH related genes. In addition to γ-glutamate cysteine ligase and the Xc-system, Nrf2 also regulates the expression of glutathione synthetase, the second enzyme required for synthesis of GSH, multidrug resistance protein transporters which export GSH from the cell and γ-glutamyl transpeptidase, the ectoenzyme responsible for cleavage of glutamate from GSH to form CysGly [28,18].

Therefore, compounds that can activate the Nrf2-ARE pathway, referred to as 'Nrf2 activators' are receiving growing attention due to their potential as GSH-boosting drugs for application to a wide range of oxidative stress related diseases [6,33,34].

This study compares four known Nrf2 activators, R-α-lipoic acid, tert-butylhydroquinone, sulforaphane and *Polygonum cuspidatum* extract containing 50% resveratrol for their effects on astroglial release of GSH and production of CysGly. R-α-lipoic acid (LA) is a naturally occurring dithiol compound with a broad neuroprotective capacity that appears to slow cognitive decline in AD patients [13]. Sulforaphane (SFN) is an organosulphur compound that is extracted from edible plants such as broccoli, brussels sprouts and cabbage and has been described as possessing anticarcinogenic, anti-inflammatory and antioxidant properties [21]. The roots of *Polygonum cuspidatum* (PC), commonly known as Japanese knotweed, are used in Traditional Chinese medicine for treatment of inflammatory diseases, hepatitis and tumours [7]. *Polygonum cuspidatum* contains resveratrol (trans-3,5,4′-trihydroxystilbene), a polyphenolic compound that has gained wide attention due to its purported anti-cancer properties [16]. Tert-butylhydroquinone (TBHQ) is an aromatic organic compound, which is used as a synthetic food grade antioxidant.

Although all four of these compounds have previously been shown to increase intracellular GSH synthesis via the Nrf2-ARE pathway [17,23,27,29], this is the first study to investigate the effect of Nrf2 activators on astrocytic production of the neuronal GSH substrate, CysGly. Furthermore, we also monitored the effect of Nrf2-activation on the levels of homocysteine (HCys), a thiol compound that is metabolically related to GSH, but is toxic to neurons at elevated levels [20,35].

Materials and methods

Cell culture and experimental protocols

The U373-MG human astrocytoma cell line was kindly provided by Dr. Peter Lock (The Royal Melbourne Hospital, Australia). U373 cells were maintained in Dulbeccos's Modified Eagle Medium (DMEM) containing 25 mM glucose, supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 2.6 μg/ml fungizone, 200 mM glutamine and 5% foetal bovine serum (FBS). Cells were grown in 175 cm^2 tissue culture flasks and incubated at 37 °C in 5% CO_2. Cells were harvested with a solution containing 0.05% trypsin and 0.02% EDTA in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and seeded into 96 well, flat-bottom, tissue culture plates at a density of 9×10^3 cells/well. FBS concentration was reduced to 3% to minimise the proliferation and the total volume of media in each well was 100 μl. After 24 h incubation, the media was replaced with fresh media containing different concentrations of potential GSH booster drugs for 48 h. All cell culture materials were from Invitrogen (Mulgrave, Australia).

Preparation of "GSH booster" drug solutions

LA, TBHQ and SFN were dissolved in 10% ethanol to produce stock solutions of 1 mg/ml. *Polygonum cuspidatum* extract (standardised to contain 50% resveratrol: PA-Res) was dissolved in dimethylsulphoxide to produce a stock solution of 100 mg/ml. Stock solutions were diluted in DMEM to give concentrations of 12.5–50 μM, 2.5–10 μM, 2.5–10 μM and 1–3.9 μg/ml of LA, TBHQ, SFN and PA-Res, respectively.

Analysis of cell viability

Cell viability was assessed in terms of the metabolic capability of cells to convert the fluorogenic redox indicator, resazurin, into its highly fluorescent product, resorufin. A modified version of the resazurin-reduction assay was used [5]. Resazurin was dissolved in PBS to give a concentration of 0.001% (w/v). This solution was sterile filtered (0.22 μm), protected from light with aluminium foil and stored at 4 °C for up to six months. To determine cell viability, incubation media was removed from wells and replaced with 100 μl of resazurin solution. Plates were incubated at 37 °C, with 5% CO_2 for 45 min and then fluorescence was measured with excitation at 530 nm and emission at 590 nm in a POLARstar Omega microplate reader (BMG Labtech). For every plate, background fluorescence determined in cell-free wells was subtracted from all wells, and values were expressed as a percentage of untreated control cells.

Determination of extracellular GSH and related thiols by high performance liquid chromatography and fluorescence detection

A Dionex HPLC system consisting of an ASI-100 automated sample injector, a P680 solvent pump, a TCC-100 thermostatted column compartment and an RF-2000 fluorescence detector was used for all chromatographic analyses. The system was equipped with a Luna C18(2) column (150 mm × 4.6 mm id, 3 μm) protected by a SecurityGuardC18 Cartridge (4.0 mm × 3.0 mm) in a SecurityGuardCartridge Holder supplied by Phenomenex. The Chromeleon 6.8 Chromatography Data System from Dionex was used to control instruments, acquire data and quantify peak areas.

Detection of thiols was performed as described previously [31]. Briefly, media taken from U373 cells treated for 48 h with LA, TBHQ, SFN or PC-Res was centrifuged at 200*g* for 5 min at 4 °C to pellet cellular debris. Following centrifugation, the supernatant was mixed with an equal volume of 1% 5-sulfosalicylic acid containing 1 mM EDTA, centrifuged at 14,000*g* for 10 min at 4 °C to precipitate protein, and the resulting supernatant placed in fresh tubes and stored at −80 °C until analysis. Upon thawing of samples and standard solutions, fresh microcentrifuge vials were placed in a heating block at 35 °C and 50 μl of sample or standard added. To reduce all disulphide bonds, 30 μl of a 1 mM solution of the reducing agent Tris(2-carboxyethyl)phosphine hydrochloride was added. For the derivatisation reaction, vials were incubated for 5 min at 35 °C before the addition of 100 μl of borate buffer (0.1 M, pH 9.3, with 1 mM EDTA) and 30 μl of the derivatising agent 4-fluoro-7-aminosulfonylbenzofurazan (ABD-F; Novachem) (1 mg/ml in 0.1 M borate buffer). Samples were incubated at 35 °C

for 10 min, before the reaction was stopped by addition of 50 μl of 2 M hydrochloric acid. Vials were then centrifuged at 14,000*g* for 5 min at 4 °C in order to pellet any particulates that could potentially damage the HPLC system. Supernatants were placed into fresh vials and loaded into an autosampler. The autosampler maintained sample temperatures at 8 °C to prevent evaporation and injected 10 μl aliquots for analysis. The mobile phase used for separation of ABD-F-derivatised thiols was 0.1 M acetate buffer (pH 4)-methanol [86:14]. An isocratic programme with a flow rate of 1 ml/min was used and column temperature was maintained at 35 °C. The fluorescence detector was set to an excitation and emission wavelength of 390 nm and 510 nm, respectively, with high level sensitivity.

Statistics

Data presented are the mean of three independent experiments and error bars denote standard deviation (SD). Significant differences were assessed by one-way ANOVA with Dunnett's post hoc tests and shown as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

U373 cell viability was assessed using the resazurin assay after 48 h treatment with increasing concentrations of LA, TBHQ, SFN and PC-Res to enable selection of non-toxic concentrations for further experiments (data not shown). To test the effect of Nrf2 activation on the release of total extracellular GSH and its derivatives, U373 astroglial cells were treated with 12.5–50 μM LA, 2.5–10 μM TBHQ, 2.5–10 μM SFN or 1–3.9 μg/ml of PC-Res for 48 h. The concentrations of total extracellular GSH, CysGly and HCys in the media were then determined by HPLC with fluorescence detection.

As shown in Fig. 1, GSH levels increased dose-dependently in response to all four drugs. SFN produced the most potent effect, increasing GSH by up to 2.4-fold compared to control cells. PC-Res, the second most potent drug, increased GSH up to 1.6-fold, followed by TBHQ (1.5-fold) and LA (1.4-fold).

Extracellular GSH is processed by the ectoenzyme, γ-glutamyl transpeptidase, to form CysGly, an important substrate for neuronal GSH synthesis. Therefore, we tested whether Nrf2 activation of U373 cells resulted in elevated CysGly in the media. Once again SFN produced the most potent effect, increasing CysGly by up to 1.7-fold, compared to control cells. TBHQ and PC-Res both induced fold increases of 1.3, followed by LA with a fold increase of 1.2 (Fig. 2).

While the order of drug potency remained almost the same for boosting GSH and CysGly, all drugs were able to increase GSH levels between 1.2 and 1.4 times more than they were able to increase CysGly (Table 1).

HCys is another thiol compound that is metabolically related to GSH, however it has been shown to be toxic to neurons at elevated concentrations [20,35]. We have previously shown that inflammation-stressed astroglial cells increase their release of HCys [30]. Therefore, we were also interested in whether Nrf2 activation resulted in increased HCys in the media. When HCys was measured in the media of cells treated with 12.5–50 μM LA, 2.5–10 μM TBHQ, 2.5–10 μM SFN or 1–3.9 μg/ml PC-Res, and in non-treated control cells. Although a trend for HCys increase for LA was observed, no significant differences were found between any of the conditions (data not shown, but fold-changes are summarised in Table 1).

Discussion

The main aim of this study was to determine whether activation of Nrf2 in astrocytes results in increased GSH release by astrocytes as well as elevated levels of extracellular CysGly. Treatment of U373 cells with four known Nrf2 activators, LA, TBHQ, SFN and PA-Res, resulted in elevated levels of both GSH and CysGly (not significant for PC-Res) in the media. For both thiols, SFN

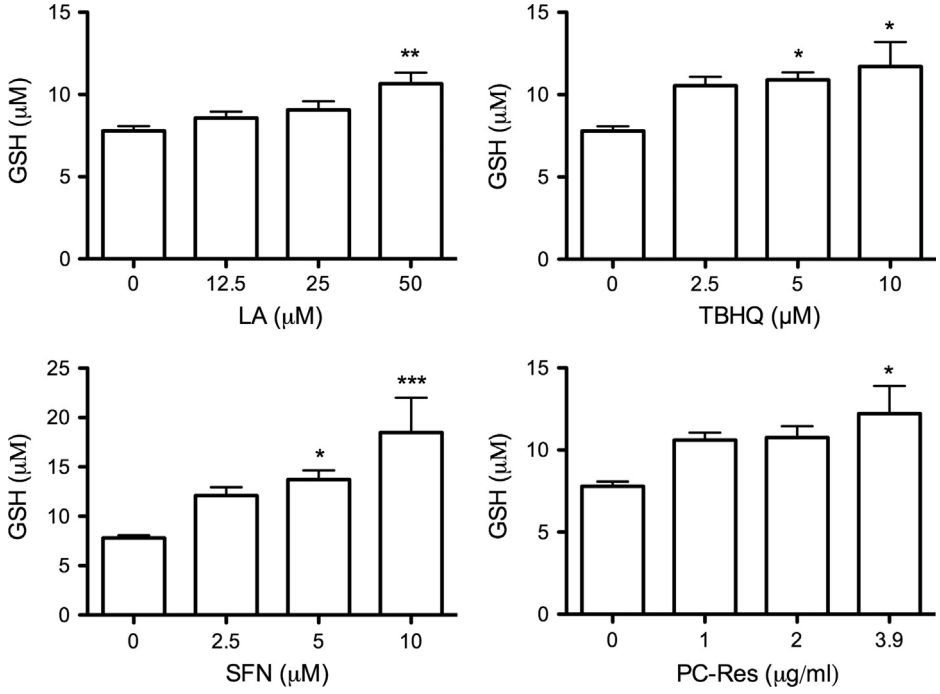

Fig. 1. Nrf2 activators increased extracellular GSH levels. U373 cells were treated with R-lipoic acid (LA), tert-butylhydroquinone (TBHQ), sulforaphane (SFN) or *Polygonum cuspidatum* containing 50% resveratrol (PC-Res) for 48 h. The concentration of glutathione (GSH) in the media was then determined by HPLC with fluorescence detection. Data points represent mean values from three independent experiments, while error bars represent SD. * and *** designate significant differences ($p < 0.05$ and $p < 0.001$) to the non-treated control.

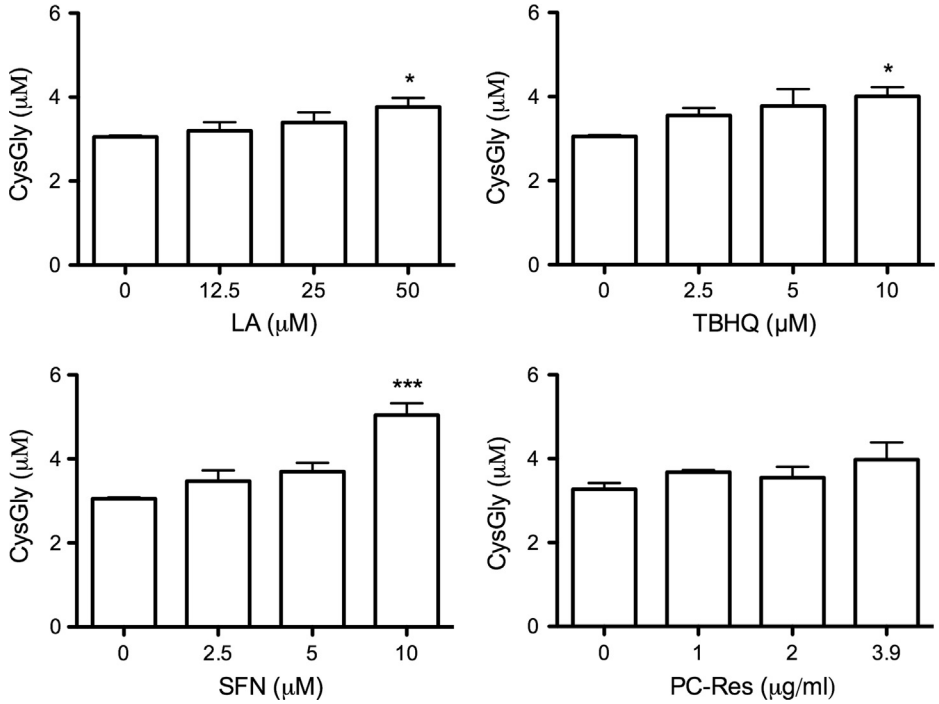

Fig. 2. Nrf2 activators increase extracellular CysGly levels. U373 cells were treated with R-lipoic acid (LA), tert-butylhydroquinone (TBHQ), sulforaphane (SFN) or *Polygonum cuspidatum* containing 50% resveratrol (PC-Res) for 48 h. The concentration of cysteinylglycine (CysGly) in the media was then determined by HPLC with fluorescence detection. Data points represent mean values from three independent experiments, while error bars represent SD. * and *** designate significant differences ($p < 0.05$ and $p < 0.001$) to the non-treated control.

Table 1
Fold differences of thiol concentrations in media between cells treated with highest, non-toxic concentrations of drugs and non-treated control cells.

Drugs	GSH	CysGly	Hcys	Ratio GSH:CysGly[a]
LA	1.4**	1.2*	1.6	1.2
TBHQ	1.5**	1.3*	0.9	1.2
SFN	2.4**	1.7**	1.0	1.4
PC-Res	1.6**	1.3	1.0	1.2

Concentrations of lipoic acid (LA), tert-butylhydroquinone (TBHQ), sulforaphane (SFN) and *Polygonum cuspidatum* extract containing 50% resveratrol (PC-Res) used to treat cells were 50 μM, 10 μM, 10 μM and 3.9 μg/ml.

[a] Ratio represents fold change in glutathione (GSH) divided by fold change in cysteinylglycine (CysGly).

* Designate significant differences ($p < 0.05$) to the non-treated control.

** Designate significant differences ($p < 0.001$) to the non-treated control.

followed by TBHQ, produced the most potent effects. When comparing the extent of the increase in GSH compared to the extent of the increase in CysGly, a larger increase was seen for GSH compared to CysGly in all four cases. It showed that, although in total more CysGly is produced by Nrf2 activated cells, a smaller percentage of extracellular GSH is being converted to CysGly in the Nrf2 activated cells compared to control cells. This finding is supported by a publication from 1996, which showed that γ-glutamyl transpeptidase (GGT) and γ-glutamate cysteine ligase (GCL) are differentially enhanced by TBHQ in rat lung epithelial L2 cells. The highest mRNA level of GGT occurred after 12 h treatment with 50 μM TBHQ, after which it decreased back to the control level by 24 h. On the other hand, GCL-mRNA level peaked after 6 h treatment with 50 μM TBHQ but was still significantly elevated after 24 h. Under the same conditions, GCL activity increased significantly after 6 h, whereas an increase in GGT was not observed until after 16 h. From nuclear run-on experiments that confirmed that the increase in GCL-mRNA, but not GGT-mRNA, was due to increased transcription, the authors suggested that the increase in GGT-mRNA probably results from a decreased degradation rate [21].

Importantly, despite a reduced rate of increase compared to extracellular GSH, significant increases in total CysGly were observed for SFN, TBHQ and LA, therefore suggesting their suitability for use as GSH booster drugs, capable of increasing delivery of GSH precursors to neurons.

When levels of HCys were analysed in media collected from cultures of U373 cells treated with LA, TBHQ, SFN and PC-Res, no changes in HCys levels were observed. HCys, a sulphydrul-containing amino acid that is metabolically linked to GSH via the transulfuration pathway is recognised as an independent risk factor for a variety of chronic diseases, including AD [11,19,22,26]. It has previously been shown that HCys exported from astrocytes is harmful to adjacent neurons through the activation of neuronal NMDA-type glutamatergic receptors and induction of oxidative stress and apoptosis [1,14,20]. The absence of an increase in HCys levels observed in response to the selected Nrf2 activators can therefore be considered as a positive outcome.

Conclusions

Based on the results presented here, LA, TBHQ, and especially SFN increase astroglial provision of GSH and CysGly, without rising extracellular levels of HCys. They therefore represent promising candidates as Nrf2-activation based drugs (if therapeutic concentration can be achieved in the target tissue) for the treatment of AD and related diseases. Additionally, based on our observation that Nrf2 activation in astroyctes increased CysGly in the media, but at a slightly reduced rate compared to GSH, co-application of direct neuronal GSH precursors such as CysGly or other cysteine homologues might also be useful.

Submission declaration

This work has not been published previously or submitted elsewhere.

This work was carried out in accordance with the Code of Ethics of the World Medical Association.

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