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Enhanced Glutathione Efflux from Astrocytes in Culture by Low Extracellular Ca^{2+} and Curcumin

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Abstract

Efflux of glutathione (GSH) from astrocytes has been suggested as a key factor for neuroprotection by astrocytes. Here we evaluated if the Nrf2 activator curcumin affects basal and stimulated (Ca^{2+} omission) GSH efflux from cultures of astroglial cells. Stimulated efflux of GSH was observed at medium concentration of 0, 0.1 mM Ca^{2+}, but not at 0.2 or 0.3 mM Ca^{2+}. Astroglia treated with 30 μM curcumin increased the cellular content of GSH in parallel with elevated basal and stimulated efflux. Conversely treatment with buthionine sulfoximine lowered efflux of GSH. The efflux stimulated by Ca^{2+}- omission was not affected by the P2X7-receptor antagonist Blue Brilliant G (100 nM) or the pannexin mimetic/blocking peptide ^{10}Panx1 but inhibited by the gap junction blocker carbenoxolone (100 μM) and a hemichannel blocker Gap26 (300 μM). RNAi directed against Nrf2 partly inhibited the effect of curcumin. The results show that elevated cellular GSH by curcumin treatment enhance efflux from astroglial cells, a process which appear to be a prerequisite for astroglial mediated neuroprotection.

Keywords

Astrocyte; Glutathione; Connexin 43; Nrf2; Curcumin

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Introduction

Astrocytes protect neurons in several ways. One route is via a glutathione (GSH) "shuttle" that involves synthesis and efflux of glutathione from astrocytes [1]. GSH is then catabolized in the extracellular space by astrocytic γ-glutamyltranspeptidase, a reaction that yields a γ-glutamyl-dipeptide amino acid and cysteinylglycine [1]. This dipeptide is taken up or broken down to cysteine and glycine [2]. Cysteine is a substrate for the EAAC1 transporter and is used intracellularly for neuronal GSH synthesis [3]. Interestingly and importantly from a potential therapeutic standpoint this intricate astrocytic defense system can be stimulated. Thus, it has been demonstrated that tert-butylhydroquinione via the transcription factor NF-E2-related factor 2 (Nrf2) and the antioxidant response element (ARE) induce the expression of a number of genes involved in the GSH defence system described above [4]. This system was recently elegantly shown to be neuroprotective in vivo against experimental amyotropic lateral sclerosis (ALS) by Nrf2 overexpression selectively in astroglial cells [5]. The GSH efflux is thus a key in this defense system. The multidrug resistance protein 1 is one efflux pathway for GSH which is upregulated by tert-butylhydroquinione [4, 6]. Another system for GSH efflux is inhibited by gap junction blockers and extracellular Ca^{2+}. The system most likely consists of half gap junctions, termed connexons or hemichannels [7, 8]. As extracellular Ca^{2+} is decreased in situations like ischemia [9] efflux of GSH via such Ca^{2+}-regulated astroglial hemichannels could be one route to supply neurons with the rate-limiting GSH precursor cysteine. Here we have further characterized the basal and stimulated efflux of GSH by omission of Ca^{2+} when its synthesis is increased or decreased. The γ-glutamylcysteine synthetase inhibitor buthionine sulfoximine (BSO) was used to reduce the intracellular GSH concentration and and curcumin to stimulate synthesis of GSH via increased expression of genes like γ-glutamylcysteine synthetase [10]. The use of curcumin was based on earlier studies demonstrating increased levels of GSH and the synthesizing enzymes in cell lines and primary cultures of astrocytes (see [10]).

Experimental Procedures

Materials

Eagle's minimum essential medium with Earle's salts (MEM), penicillin–streptomycin (PEST), amino acids, vitamins, and L-glutamine were purchased from Invitrogen (Merelbeke, Belgium), and fetal calf serum from Merck (Berlin, Germany). The artificial cerebrospinal fluid (ACSF) consisted of (mM): NaCl (128), KCl (3), $CaCl_2$ (2), $MgSO_4$ (1.2), KH_2PO_4 (0.4), $NaHCO_3$ (25) and D-glucose (10) and Gey's balanced salt solution of (mM): NaCl (119), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1) $MgSO_4$ (0.3), KH_2PO_4 (0.2), $NaHCO_3$ (27), Na_2HPO_4 (0.67) and D-glucose (5.5). In experiments were the effects of low extracellular levels of Ca^{2+} was evaluated $CaCl_2$ was omitted from the medium or $CaCl_2$ was added at the indicated concentrations. Chemical analysis of the Ca^{2+} concentration in the respective media was not performed. The solutions were equilibrated with gas mixtures (see further below) containing 5% CO_2 which resulted in a pH ~7.4. All salts in ACSF were from Sigma or Merck (Darmstadt, Germany) and methanol was from Rathburn (Rathburn Chemicals Ltd, Walkersburn, UK). Carbenoxolone (CBX), BSO and Brilliant blue G (BBG) were bought from Sigma (St. Louis, MO, USA). The connexin 43 (Cx43) mimetic/blocking peptide (Gap26) (VCYDKSFPISHVR) and the pannexin1 mimetic/blocking peptide (WRQAAFVDSY), ^{10}Panx1, were synthesized by solid-phase chemistry and purified by HPLC to 95% purity (Sigma-Genosys) [11, 12]. Curcumin of 80% purity was also purchased from Sigma. Curcumin and ^{10}Panx1 were dissolved in DMSO and diluted in ACSF and ACSF/0 Ca^{2+} to a final DMSO concentration of 0.1%.

Primary Astrocyte Cultures

Primary cultures of astrocytes were prepared from the hippocampi of newborn (P1-P2) Sprague–Dawley rats as previously described [13]. In brief, the rats were decapitated and the hippocampi were carefully dissected. The tissue was mechanically passed through a nylon mesh (80 μm mesh size) into culture medium consisting of MEM supplemented to the following composition: 20% (v/v) fetal calf serum, 1% penicillin–streptomycin, 1.6 time the concentrations of amino acids and 3.2 times the concentration of vitamins (in comparison to MEM), 1.6 mM L-glutamine, 7.15 mM glucose and 48.5 mM $NaHCO_3$. The cells were grown in 35 mM wells at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed after 3 days in culture and thereafter three times a week. Cells were used after 14–19 days in culture when a confluent monolayer had been formed. Curcumin and BSO were added to the culture medium 24 h prior to the experiments.

siRNA Mediated Knock-Down of Nrf2

Down regulation of the Nrf2 expression was achieved by siRNA technique as described earlier [14]. The astrocytes were transiently transfected using ON-TARGETplus SMARTpool siRNA against rat Nrf2 (Thermo Scientific Dharmacon, CO, USA). ON-TARGET plus non-targeting pool (Thermo Scientific Dharmacon, CO, USA) was used as negative control. The astrocytes were grown in 12-well plates to confluency (13–14 days in vitro). The transfection was initiated by incubating the cultures with OptiMEM (Invitrogen, CA, USA) for 30 min. Nrf2 ON-TARGETplus SMARTpool siRNA or ON-TARGET plus non-targeting pool (100 nM, final concentration) was mixed with Lipofectamine 2000 (3 μl; Invitrogen, CA, USA) in OptiMEM and incubated for 20 min prior to addition to the astrocytes (final volume 1 ml). After 5 h, the transfection mixture was replaced with OptiMEM containing 20% serum and the astrocytes were incubated for 19 h. The cultures were thereafter further incubated in normal growth medium with or without curcumin (30 μM) for another 24 h. The efficiency of the knockdown was evaluated by western blot (Fig. 6b) using α-Nrf2 antibody (dilution 1:1,000, R&D Technologies). The optical densities of Nrf2 blot was correlated to the densities of tubulin and showed a decrease in the level of Nrf2 by approximately 70% compared to untreated samples.

Efflux Experiments

The efflux experiments were carried out by incubating the cells with ACSF or ACSF with no Ca^{2+} added (400 μl) for 10 min. The fluid was then removed and filtered before immediate HPLC analysis or storage in −20°C (maximally 2 weeks). The incubation procedure was repeated 7 times (70 min in total) with Ca^{2+} omission occurring during the fourth and fifth incubation period. All inhibitors were present during a 30 min preincubation period and the entire incubation period (60 min in total before Ca^{2+} removal). All solutions were equilibrated with a gas mixture containing 5% CO_2 to reach a pH of ~7.4. Thereafter, all solutions were put in an incubator in a humidified atmosphere of 95% air and 5% CO_2 at 36.5°C for at least 30 min. After the seventh incubation, the cells were scraped into 400 μl of 0.3 M $HClO_4$ and sonicated. After centrifugation at 11,000g the supernatant was removed and filtered (Acrodisc, 0.2 μm, Pall Corporation, Ann Arbor, MI, USA). The supernatant was used to determine the cellular content of glutathione and amino acids. The protein pellet was dissolved in 100 μl 2% SDS and the protein content was measured using the bicinchoninic acid method [15].

HPLC Determination of Amino Acids and GSH

All chromatography was performed using a Varian 5000 or 5500 HPLC pump coupled to a fluorescence detector (Schoeffel FS 970) for detection of the fluorescence intensities of OPA-derivatives of glutathione and amino acid (see below for details). Data were processed

with Millennium and Maxima software (Waters Corporation, Milford, MI, USA). All separations were performed at room temperature. Sample injection was made using a Waters 717 autosampler.

GSH and amino acids were determined using OPA derivatization and fluorescence detection essentially as described earlier [16]. A solution of β-mercaptoethanol, Na_2-EDTA and NaN_3 (final concentration 20, 1, 5 mM, respectively) was added to the samples and standards to keep GSH in its reduced form and prevent bacterial growth. The OPA-solution was prepared weekly and consisted of OPA (40 mg) dissolved in methanol (400 μl), β-mercaptoethanol (40 μl), borate buffer (2.0 ml, 0.8 M, pH 12) and H_2O (1.6 ml). Every 2 days β-mercaptoethanol (10 μl) was added to the solution. Amino acids were derivatized (25 μl of sample mixed with 25 μl OPA solution) in the autosampler before injection. The amino acid derivatives were separated on a Nucleosil C18 column (200 × 4.6 mM; Macherey-Nagel, Germany) with a mobile phase consisting of NaH_2PO_4 (50 mM, pH 5.28) and methanol in a gradient from 25 to 95% methanol. A flow rate of 1 ml/min was used. Detection was carried out by excitation at 333 nm and emission over 418 nm. To test for possible interactions with GSH, standard solutions with or without drugs were analysed. No differences in the peak heights of GSH were found with any of the drugs used.

Statistics

Statistical analysis was performed with SPSS software (SPSS Inc., Chicago, Illinois, USA). One-way ANOVA followed by Tukey's test for multiple comparisons and Wilcoxons signed rank test were used for statistical analysis. A p value of <0.05 was considered statistically significant. Data shown in figures are from at least 3 independent cultures and expressed as means ± SEM.

Results

The efflux profile from the primary astroglial cells by omission of Ca^{2+} was similar to that reported from organotypic cultures [8], i.e. the efflux rates of GSH, glutamate, taurine (not shown) and phosphoethanolamine were particularly elevated (Fig. 1a, b). The stimulated efflux by omission of Ca^{2+} was not affected by the P2X7- receptor antagonist Brilliant Blue G (BBG, 100 nM) or the pannexin mimetic/blocking peptide 10Panx1 (300 μM) but inhibited by the gap junction blocker carbenoxolone (100 μM) and the connexin43 mimetic/blocking peptide Gap26 (300 μM, Fig. 2).

Stimulated efflux of GSH was observed at 0.1 mM Ca^{2+}, but not at 0.2 or 0.3 mM Ca^{2+} (Fig. 3). Blocking the synthesis of GSH by adding BSO (1 mM for 24 h) to the culture medium decreased the cellular content of GSH by ~70% (Fig. 5). This treatment also decreased the basal efflux (~70% lower compared to culturing without BSO) and efflux of GSH stimulated by omission of extracellular Ca^{2+} (~85% lower compared to cells cultured without BSO) (Fig. 4a, b). The effect of BSO on basal efflux was selective for GSH, i.e. the efflux of other amino acids was unaffected. Treatment with BSO caused no change in efflux stimulated by omission of Ca^{2+} for phosphoethanolamine or taurine (not shown) but reduced that of glutamate (Fig. 4b). The cellular content of phosphoethanolamine was unchanged but that of glutamate increased by BSO treatment (Fig. 5).

Astrocytes cultured for 24 h with 30 μM curcumin increased the efflux rates of GSH in basal medium and in medium with omitted Ca^{2+} by 90 and 160%, respectively (Fig. 4a–b). The basal and stimulated efflux of GSH by Ca^{2+} omission in curcumin treated cells was dramatically reduced by carbenoxolone but notably not down to levels reached in cultures not treated with curcumin (Fig. 4a–b). The basal efflux and the efflux stimulated by Ca^{2+} omission of phosphoethanolamine and glutamate were not changed by treatment with

curcumin. Addition of curcumin to the culture medium increased the cellular content of GSH approximately 6 times whereas the levels of phosphoethanolamine and glutamate were unaffected (Fig. 5). The effects of curcumin on GSH cellular content were reduced considerably by prior treatment of the cells with siRNA against Nrf2 (Fig. 6a) whereas no effect of siRNA was observed on GSH levels in non-curcumin treated cultures.

Discussion

Stimulated GSH synthesis in astroglia is neuroprotective via a shuttle that involves GSH efflux as described in the introduction [1]. Here we characterized and showed that the efflux of GSH can be enhanced by curcumin and by low extracellular Ca^{2+}.

The stimulating effect on efflux by omission of extracellular Ca^{2+} is in line with several reports. Stimulated efflux of GSH [7, 8], glutamate [17], taurine [8, 17], NAD^+ [18] and ATP [19] has been demonstrated earlier from astroglial cells in low or nominal Ca^{2+}-free media. Most studies concerning efflux by Ca^{2+}-omission have been performed in cultures. However, similar effects on glutamate efflux have been observed in vitro from acutely prepared optic nerves of mice, indicating that this efflux pathway also exist in vivo [17]. The most likely efflux route when extracellular Ca^{2+} is reduced in the incubation medium is via connexin hemichannels. This is based on the findings that the efflux was blocked by general gap junction blockers and a more specific connexin mimetic peptide, Gap26 [20]. Further, no effect was observed using the $P2X_7$-receptor blocker BBG and the pannexin mimetic/blocking peptide ^{10}Panx1. The lack of effects of $P2X_7$-receptor blockers are in accordance with several studies [7, 8]. In addition and as far as we know none of the putative transport proteins, pannexin hemichannels, $P2X_7$-receptors, volume sensitive anion channels, voltage dependent anion channels, volume sensitive outwardly rectifying channels, cystic fibrosis transmembrane conductance regulator and vesicular release pathways are blocked by extracellular Ca^{2+}. The threshold concentration for effects on efflux of GSH was 0.1 mM Ca^{2+} whereas incubation in 0.2 or 0.3 mM Ca^{2+} did not stimulate efflux. This agrees well with an earlier study on stimulated efflux of glutamate from primary cultures of astroglia by low extracellular Ca^{2+} [17]. Low extracellular Ca^{2+}-concentrations are reached during anoxia and spreading depression [9]. It is interesting to note that the newly developed techniques for measurements of extracellular Ca^{2+} reveal that the earlier used techniques greatly underestimate the drops in Ca^{2+} [21]. It is thus possible but remains to be demonstrated that more physiological nervous activity can induce dramatic decreases in extracellular Ca^{2+} that are compatible with connexin hemichannel opening.

The treatment with curcumin was selective and strongly activated both the basal and stimulated efflux of GSH but did not increase efflux of other components measured. In curcumin treated cells both the basal efflux of GSH and the efflux stimulated by omission of Ca^{2+} were decreased by carbenoxolone. However, carbenoxolone did not reduce GSH efflux down to basal levels in curcumin treated cells. This indicates that other GSH efflux pathways that are not sensitive to carbenoxolone is/are elevated by curcumin. Interestingly multidrug resistance protein 1 is upregulated by Nrf2 [22]. The effect of curcumin on GSH levels was in our study reduced by Nrf2 directed siRNA. It therefore appears likely that the elevated basal efflux of GSH is in part due to efflux via elevated levels of multidrug resistance protein 1 [23].

Both basal and stimulated GSH efflux rates were enhanced by curcumin and reduced by lowering the intracellular GSH by buthionine sulfoximine. GSH efflux is thus closely linked to its intracellular levels. The finding agrees well with early studies by Sagara and coworkers who showed that astrocytes release GSH depending on their concentration in a wide range [24]. The discrepancy in our case between the increase in cellular level (about 6

× control) and the less prominent elevated efflux (about 2.5 × control) of GSH is mostly likely due to saturation of the efflux. This was suggested already by Sagara and coworkers who showed that the efflux of GSH was linear at low cellular levels of GSH but saturated >40 nmol/mg protein [24]. In our case treatment with curcumin increased the cellular level to about 85 nmol/mg protein. This large elevation of cellular GSH in comparison to basal cellular levels is likely due to that curcumin to a higher degree increase the expression of the modulatory subunit of γ-glutamylcysteine ligase in comparison to the catalytic subunit [10]. The high proportion of modulatory subunit will increase the Ki of glutamylcysteine ligase for GSH and lower the Km of γ-glutamylcysteine ligase for glutamate [25]. As this enzyme is rate-limiting for GSH synthesis the increase in the modulatory subunit will elevate synthesis to reach high levels of GSH [25].

The stimulated efflux pathway of phosphoethanolamine by low extracellular Ca^{2+}, appear to be the same route as for GSH [8]. Phosphoethanolamine efflux was similar in treated cultures (BSO and curcumin) compared with control cultures. This indicates that the efflux pathway that is controlled by extracellular Ca^{2+}, putatively connexin hemichannels, is unchanged by the treatments and that the altered efflux of GSH is related to changed cellular content rather than to a enhanced efficiency of the hemichannel efflux. However, and as noted above, it appears that the efflux via multidrug resistance protein 1 is upregulated by curcumin. Strangely BSO treatment increased the cellular content of glutamate but decreased efflux of glutamate stimulated by Ca^{2+} omission (but not the basal efflux). The increase in cellular glutamate is probably due to less incorporation of glutamate in γ-glutamylcysteine as the enzymatic step blocked by BSO is the ligation of glutamate and cysteine [25]. The factors behind the reduced glutamate efflux in medium with no added Ca^{2+} in BSO treated cultures is at present unknown. However, the efflux is a combination of release and uptake. One major difference between glutamate and GSH/phosphoethanolamine is the avid uptake of glutamate in contrast to GSH/phosphoethanolamine. In an earlier study no effects on the excitatory amino acid transporters were found in BSO treated primary astrocyte cultures [26] which makes elevated Na^{+}-dependent uptake a less likely explanation. Alternatively other pathways for glutamate efflux could be reduced by intracellular GSH or BSO treatment. Indeed it has been shown that BSO decrease cystine uptake [27] which would lead to decreased glutamate efflux via the Xc^{-} system.

The increase in cellular content of GSH in the present experiments is well in line with the findings that curcumin also in vivo can increase GSH levels in normal and ischemic conditions [28, 29]. The results indicate that the elevated basal GSH efflux by curcumin may be one mechanism for the neuroprotection afforded by curcumin [30]. Interestingly, the curcumin-induced but not the basal cellular content of GSH was decreased by siRNA against Nrf2. The lack of decreased basal GSH after siRNA agrees well with that Nrf2 knock-out and wild-type mice have similar GSH content in brain [31]. These findings indicate that, at least in young animals, the constitutive synthesis of GSH is independent of the Nrf2 system [31].

Overall the results shows that the intracellular level of GSH grossly determine the basal and stimulated efflux rates by Ca^{2+}-omission. From a neuroprotective standpoint this implies that increasing the astroglial content of GSH with curcumin will “feed” the neurons with the rate-limiting GSH precursor cysteine. This may be particularly important in conditions such as Parkinson's disease where a dramatic tissue drop of GSH in the substantia nigra is observed and in normal ageing which is accompanied by a decrease in brain GSH levels [32].

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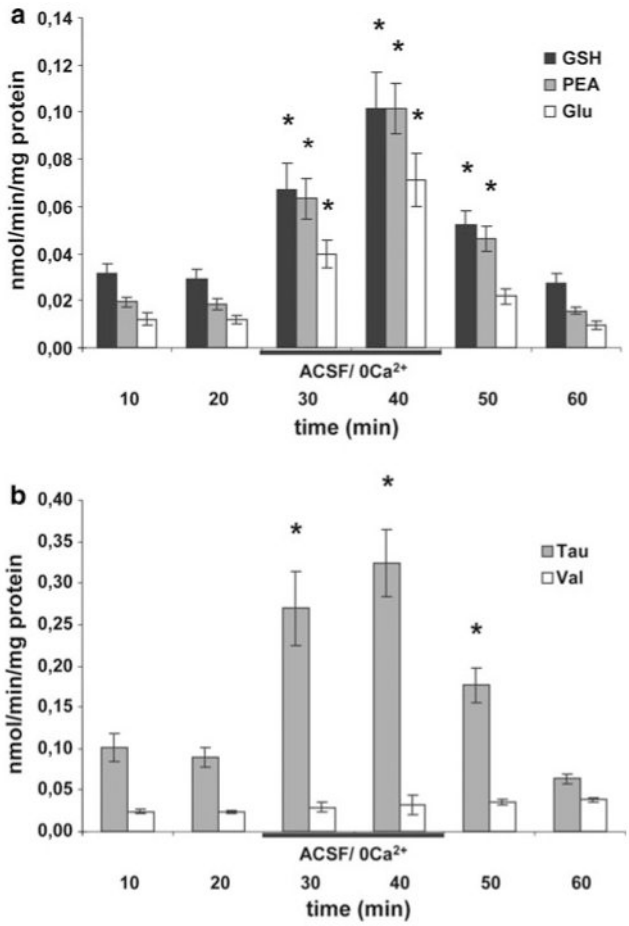

Fig. 1.

a Time course of stimulated efflux of glutathione (GSH), phosphoethanolamine (PEA) and glutamate (Glu) caused by omission of Ca^{2+}. The efflux rates reached their maxima 10 min after the introduction of ACSF/0 Ca^{2+}. **b** Time course of efflux for taurine (Tau) and valine (Val) following omission of Ca^{2+}. No change in efflux was observed for Val. Data are presented as mean efflux rate ($n = 6 \pm$ SEM). *Stars* in *a* and *b* indicate a significant difference between efflux in ACSF and ACSF/ 0 Ca^{2+} ($p < 0.05$)

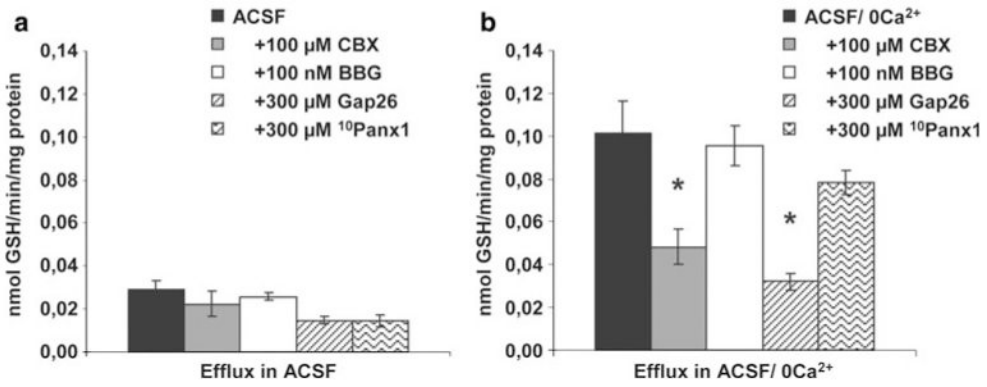

Fig. 2.

a The basal efflux of GSH in ACSF was not changed by the gap junction inhibitor carbenoxolone (CBX), the $P2X_7$-receptor antagonist Brilliant Blue G (BBG), the the connexin hemichannel mimetic/blocking peptide Gap26 or the pannexin hemichannel mimetic/blocking peptide ^{10}Panx1. **b** The gap junction blocker carbenoxolone (CBX) and the connexin hemichannel blocking peptide Gap26 significantly reduced the efflux of GSH caused by omission of Ca^{2+} while the $P2X_7$-receptor antagonist Brilliant Blue G (BBG) and the pannexin hemichannel mimetic/blocking peptide ^{10}Panx1 did not cause significant effects. Data are presented as mean efflux rate ($n = 6 \pm$ SEM). *Stars* mark a significant difference ($p < 0.05$) in efflux with inhibitors compared to efflux in ACSF/0 Ca^{2+} without inhibitors ($n = 6 \pm$ SEM)

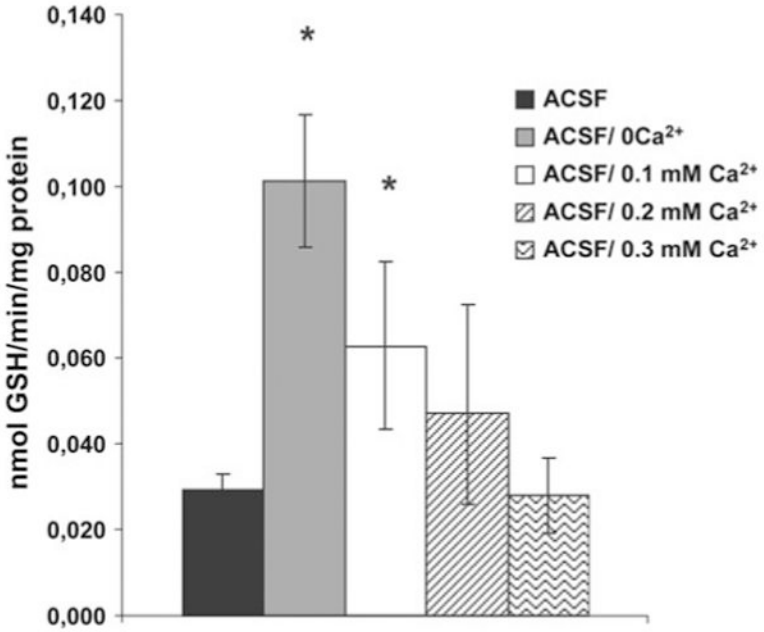

Fig. 3.

The efflux of GSH was stimulated by 0.1 mM Ca^{2+} but not by 0.2 mM Ca^{2+} or 0.3 mM Ca^{2+}. Data are presented as mean efflux rate ($n = 6 \pm$ SEM). *Stars* mark a significant different efflux ($p < 0.05$) compared to efflux in ACSF

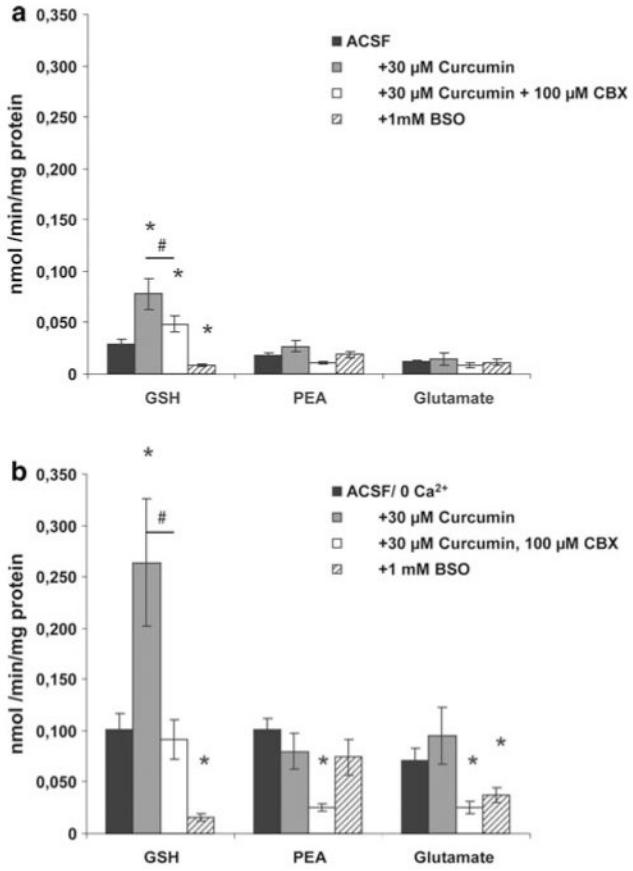

Fig. 4.

a Effects on efflux of GSH, phosphoethanolamine (PEA) and glutamate (Glu) in ACSF after treatment of the astrocyte cultures for 24 h with 30 µM curcumin or 1 mM buthionine sulfoximine (BSO). The efflux of GSH in ACSF was decreased by treatment for 24 h with BSO and increased by treatment with curcumin. The increased basal efflux of GSH in ACSF was decreased by the gap junction inhibitor carbenoxolone (CBX). No significant effects were observed for efflux of PEA or Glu. *Stars* mark significant different efflux ($p < 0.05$) by treatment for 24 h compared to no treatment. # marks significant difference between curcumin treated samples with or without carbenoxolone. **b** Effects on efflux of GSH, phosphoethanolamine (PEA) and glutamate (Glu) in ACSF/ 0 Ca^{2+} after treatment of the astrocyte cultures for 24 h with 30 µM curcumin or 1 mM buthionine sulfoximine (BSO). The stimulated efflux of GSH was decreased by treatment for 24 h with BSO and increased by treatment with curcumin. The gap junction blocker carbenoxolone (CBX) reduced the enhanced stimulated efflux of GSH in curcumin treated cultures. No significant effects by treatment with curcumin were observed on efflux of PEA or Glu in ACSF/0 Ca^{2+}. The stimulated efflux of PEA and Glu were reduced by CBX (only evaluated in curcumin treated slices). The stimulated efflux of Glu was reduced after treatment of the cultures with BSO for 24 h. Data are presented as mean efflux rate ($n = 6 \pm$ SEM). *Stars* mark a significant difference ($p < 0.05$) in efflux with inhibitors or treatment compared to efflux in ACSF/0 Ca^{2+} without inhibitors. # marks significant difference between curcumin treated samples with or without carbenoxolone

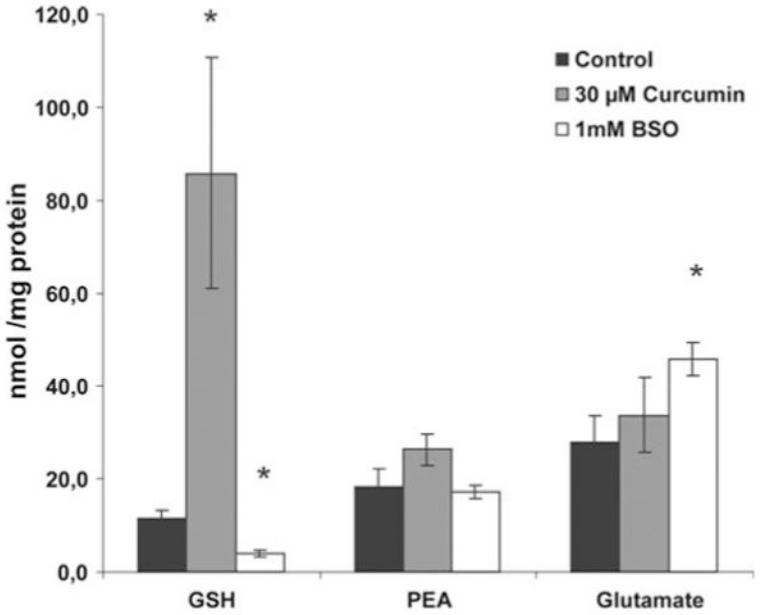

Fig. 5.

Cellular concentration of GSH, phosphoethanolamine (PEA) and glutamate (Glu) in astrocyte cultures treated for 24 h with 30 μM curcumin or 1 mM buthionine sulfoximine (BSO). The cellular concentration of GSH was increased by treatment for 24 h with 30 μM curcumin and decreased by treatment with 1 mM BSO. Treatment with BSO also increased the cellular concentration of Glu. *Stars* mark a significant different efflux ($p < 0.05$) compared to control value

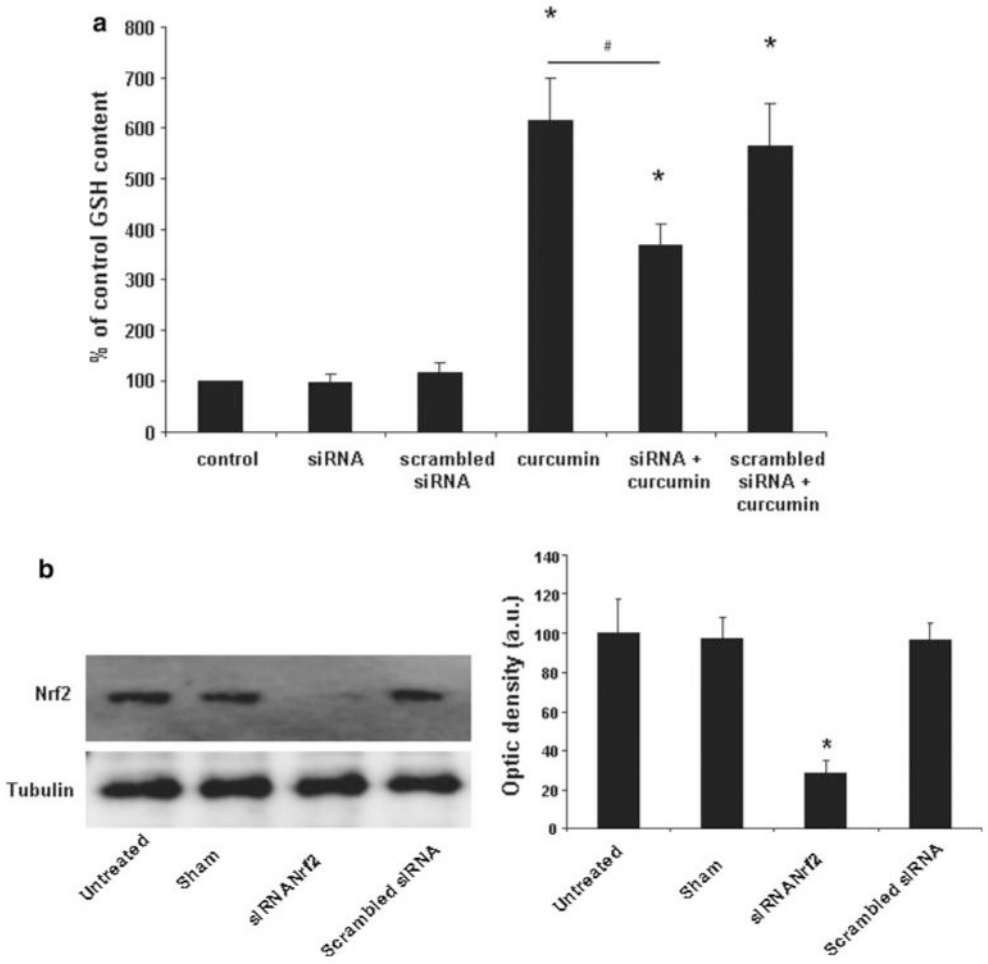

Fig. 6.

a The effect of curcumin (30 μM) on intracellular GSH concentration is significantly reduced by siRNA mediated knockdown of Nrf2. No effect was observed on basal levels of GSH or when the cells were transfected with scrambled siRNA. Values are given as percent relative to GSH concentration in the control group ($n = 8 \pm$ SEM). The level of GSH in cells treated with 30 μM curcumin after treatment with siRNA against Nrf2 was significantly lower (Wilcoxon signed rank test, $p < 0.05$) compared to the levels induced by curcumin in non-subjected cells and to levels in cells treated with a scrambled siRNA. *Stars* mark a significant difference ($p < 0.05$) compared to control and # marks significant difference between curcumin and siRNA treated cultures. **b** *Left*: Treatment with siRNA directed against Nrf2 lowered the expression of the Nrf-2 protein by approximately 70%. *Right*: Densitometric analysis of Nrf2 protein expression in astrocyte cultures treated with siRNA directed against Nrf-2. Data are plotted as ratio of the Nrf2/tubulin obtained in each condition