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N-acetyl-L-cysteine as a source of sulfane sulfur in astrocytoma and astrocyte cultures: correlations with cell proliferation

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Summary. N-acetyl-L-cysteine (NAC), a precursor of L-cysteine, not only elevates the level of glutathione in both astrocytoma and astrocyte cultures, but also affects the cellular level of sulfane sulfur. Astrocytoma cells were investigated using the stable U373 human cell line. In the U373 cells, N-acetyl-L-cysteine, depending on the concentration in the culture medium and culture duration, either elevated or diminished the level of sulfane sulfur, and this was respectively accompanied by decreased or increased cellular proliferation. In murine astrocytes, in turn, NAC was capable of lowering the level of sulfane sulfur and in this way decreased cellular proliferation. It seems that normal (astrocyte) and transformed (astrocytoma) cells differed in their reaction to NAC in the culture medium. The effect of N-acetyl-L-cysteine on astrocytoma cells was advantageous in that it inhibited their proliferation through the elevation of the level of sulfane sulfur.

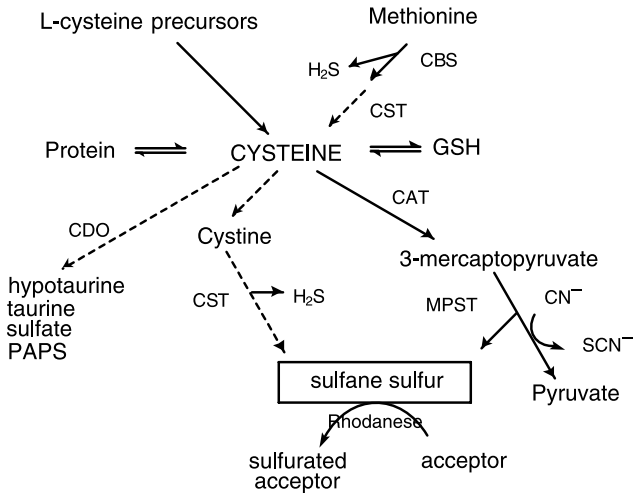
Keywords: Astrocytes – U373 cells – N-acetyl-L-cysteine – 3-Mercaptopyruvate sulfurtransferase – Rhodanese – Sulfane sulfur

Introduction

Non-oxidative L-cysteine metabolism (L-cysteine desulfuration) is the main source of metabolically active sulfane sulfur atoms (i.e. divalent sulfur atoms having a reduced oxidation state, bonded only to other sulfur, commonly existing in mammalian sera and tissue) (Ogasawara et al., 1993, 1994). 3-Mercaptopyruvate sulfurtransferase (MPST, EC 2.8.1.2), rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1) and cystathionase (cystathionine γ-lyase, CST, EC 4.4.1.1) are well-known enzymes participating in the formation of sulfane sulfur-containing compounds and transferring their labile sulfur atoms to various acceptors (Scheme 1). Westley et al. (1983) introduced the concept of a metabolic pool of sulfane sulfur for biosynthetic purposes (e.g., for forming iron sulfur proteins and for detoxification of such compounds as, for example, cyanide) (Westley, 1973, 1980; Beinert, 2000; Liew and Shaw, 2005). Furthermore, Toohey (1989) reviewed the topic and stated that sulfane sulfur affected biological systems (cell proliferation, protein function, immune system-enhancement and anti-cancer effects), and suggested that it had a natural regulatory function. The antioxidant potential of sulfane sulfur and rhodanese has been also recently investigated (Ogasawara et al., 1999; Nandi et al., 2000; Wróbel et al., 2004). Although the biological role of reduced sulfur, such as sulfane sulfur, is not completely understood, according to Toohey (1989) malignant cell proliferation may be related to a deficiency of sulfane sulfur and the uncontrolled operation of a set of enzymes normally inactivated by sulfane sulfur.

On the basis of previous investigations performed in Ehrlich ascites tumor cells (Włodek et al., 1993) or in sarcoma (Frendo et al., 2002), one may expect that metabolic processes leading to an increased sulfane sulfur level are inhibited in neoplastic cells. Reduced activity of rhodanese was also found in cancerous tissues of the stomach and the lung (Jamshidzadeh et al., 2001).

Precursors of L-cysteine, both in normal and in neoplastic cells, may affect the level of non-protein sulfhydryl groups, sulfane sulfur compounds and the activity of sulfurtransferases involved in L-cysteine desulfuration (Roberts et al., 1998; Roberts and Francetic, 1991; Wróbel et al., 1997; Wróbel and Włodek, 1997) (Scheme 1). Verifying whether N-acetyl-L-cysteine (NAC), a well-known and therapeutically employed precursor of cysteine (Lyn, 2000), may affect the sulfane sulfur level in normal and neoplastic brain cells through stimulation of the activity of MPST is a novel approach to the problem of inhibiting neoplastic cell proliferation. It was our intention to



Scheme 1. Metabolic pathways involving L-cysteine. *CBS* Cystathionine beta-synthase, *CAT* cyteine aminotransferase, *CDO* cysteine dioxygenase

ascertain a relationship between the level of sulfane sulfur, the activity of enzymes participating in its generation (MPST, cystathionase) and metabolism (rhodanese), and cellular proliferation. Should a decreased sulfane sulfur level be confirmed in neoplastic brain cells (U373 cells) in comparison with normal brain cells (astrocytes), this would demonstrate that cysteine precursors – through restoration of the sulfane sulfur level to values characteristic of normal cells – are able to play a part in the inhibition of neoplastic cell proliferation, which would be of accessory therapeutic importance.

Materials and methods

Sources of chemicals

Folin-Ciocialteau reagent, NADPH (Na_4), NADH, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), glutathione reductase, lactate dehydrogenase (LDH), pyridoxal phosphate (PLP), 1,4-dithio-bis-(2-nitrobenzoic acid) (DTT), N-ethylmaleimid (NEM), N-acetyl-L-cysteine (NAC), poly-L-lysine, trypsin, sodium pyruvate, DMEM (Dulbecco's Modified Eagle Medium) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Fetal bovine serum was obtained from GIBCO Laboratories (Grand Island, NY, USA), potassium cyanide (KCN) from Merck (Darmstadt, Germany), sodium 3-mercaptopyruvate from Flucka Chemie GmbH. The Cytotoxicity Detection Kit (LDH) and Cell Proliferation ELISA, BrdU (colorimetric) test were obtained from Roche Applied Science. All other chemicals were of reagent grade and purchased from common commercial suppliers.

Cell cultures

All experiments were performed using the human astrocytoma U373 cell line and the murine cortical astrocyte primary culture. Human astrocytoma U373 cells were grown in monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), in plastic culture dishes (100 mm in diameter), at 37 °C, in a humidified atmosphere containing 5% CO_2. After the tripsinization (0.25% trypsin/EDTA), the cells were diluted with complete medium (DMEM with 10% FBS) (Ponten and Macintyre, 1968). Murine cortical astrocyte primary cultures established from 1-day-old pups (Swiss Albino strain) were grown to confluence in DMEM supplemented with 10% fetal bovine serum, pyruvate (110 mg/l), glucose (4500 mg/l), and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), at 37 °C, in a humidified atmosphere containing 5% CO_2. The cells were plated in poly-L-lysine-coated flasks (75 cm^2) (McCarthy and de Vellis, 1980), and were collected after 1–2 passages. All the cultures used in the experiments were about 2-week old.

Treatment of cells with NAC

The cells were seeded at 1 mln/100 mm dish the day before treatment. NAC (0.5 and 1 mM) was dissolved in cell culture media and neutralized when necessary, and filtered through a 0.22-µm filter for sterilization. The cells were incubated for 6, 12, 24, 48 or 72 h and then washed three times with 3 ml of cold PBS (10 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl) to completely remove sulfhydryl-group-containing compounds that might interfere with the glutathione assay. Then the cells were harvested in cold PBS, centrifuged at 5000 rpm at 4 °C during 10 min and solubilized in phosphate buffer, pH 7.5 for homogenization.

Cell homogenization

U373 cells and astrocytes ($1–7 \times 10^6$) were suspended in 0.1 M phosphate buffer pH 7.5, in the proportion 1 ml cells/0.04 ml of the buffer, sonicated 3×5 sec at 4 °C (Bandelin Sonoplus GM 70). After centrifugation at 5000 rpm at 4 °C for 10 min, the supernatant was used for the determination of protein concentration, sulfane sulfur levels and the activity of rhodanese, MPST and CST. For glutathione determinations, the homogenate was deproteinized by adding 5% trichloroacetic acid in the proportion 1:1 and centrifuged for 10 min at 3000 rpm.

Enzyme assay

MPST activity was assayed according to the method of Valentine and Frankelfeld (Valentine and Frankenfeld, 1974), following a procedure described in our earlier paper (Wróbel et al., 1997). The enzyme activity was expressed as nmoles of pyruvate produced during 1 min incubation at 37 °C per 1 mg of protein. Rhodanese activity was assayed by Sörbo's method (1955), following a procedure described in Wróbel et al. (1997). The enzyme activity was expressed as nanomoles SCN^- formed during 1 min incubation at 20 °C per 1 mg of protein. Cystathionase activity was determined by Matsuo and Greenberg's method (1966), modified as described in Czubak et al. (2002). The enzyme activity was expressed as nanomoles α-ketobutyrate formed during 1 min incubation at 37 °C per 1 mg of protein.

Sulfane sulfur

Sulfane sulfur was determined by the method of Wood (1987), based on cold cyanolysis and colorimetric detection of ferric thiocyanate complex ion, protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard, and the cellular glutathione content was determined by a modification of enzymatic assay originally described by Tietze (1969). The Tietze method measures the rate of reduction of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase. Since NAC also reacts with DTNB to generate the colored 5-thio-2-nitrobenzoic acid (TNB) compound, it was important to remove all possible external sulfhydryl compounds before assay.

Cell proliferation

Cell proliferation was examined using the Cell Proliferation ELISA, BrdU (colorimetric) test (Roche Applied Science). The assay was performed

according to the manufacturer's protocol. Each test (or its paired control) was performed in triplicate for each experiment. For the determination of cellular proliferation, the cells were seeded into 96-well plates at a concentration of $1.5–2 \times 10^3$ cells/well (U373 cells), or $1.5–5 \times 10^3$ cells/well (astrocytes) in DMEM supplemented as reported above. Twenty-four hours after the initial seeding, the culture medium was replaced with 100 µl of complete medium (control cultures) or 100 µl of medium containing tested NAC concentrations (0.25, 0.5, 0.75, 1, 1.5, 2, and 5 mM) and the plates were cultured for various time intervals (U373 cells: 6, 12, 24, and 48 h; astrocytes: 48 and 72 h).

Determination of cell viability

Cell viability was investigated by measuring the leakage of LDH from dead or dying cells into the culture medium using the Cytotoxicity Detection Kit. Briefly, the cells were cultured in 96-well plates at a density of $1–2 \times 10^3$ U373 cells per well or $1.5–5 \times 10^3$ astrocytes per well. After treatment, LDH was determined spectrophotometrically by measuring its activity in an aliquot of cell-free medium. One hundred microliters of the culture medium collected from the dishes after the incubation of the cells were added to 100 µl of the incubation mixture and incubated for 30 min. at room temperature, in the dark. After the incubation, 50 µl of 1 M HCl was added and the absorbance was measured with a microculture plate reader at 490 nm. The leakage was calculated as a percentage of total activity after lysis of the cells by 1% Triton X-100.

Presentation data and statistical analysis

Each group consisted of 3–4 culture dishes per experiment for each time point studied. The experiments were performed in triplicate or quadruplicate, and the cultures were taken from 4–7 separate seedings. The data are presented as means ± standard deviation from the individual values measured. The statistical significance of the antiproliferative effect of NAC was determined using the Student's *t*-test. $p < 0.05$ was considered significant.

Results

Both cell types showed trace or no γ-cystathionase activity. Table 1 presents the activity of rhodanese and MPST in astrocytes and the U373 cells. Compared with astrocytes, the U373 cells showed rhodanese activity decreased 3 times and MPST activity decreased about 5 times. In consequence, they showed also significantly lower ($p < 0.001$) sulfane sulfur levels, the decrease amounting to approximately 20%. It should be noted that glutathione levels were similar in both the U373 cells and astrocytes.

Table 1. Sulfurtransferases activity, sulfane sulfur and glutathione levels in mouse astrocytes and astrocytoma U373 cells

Control cells	MPST	Rhodanese	Sulfane sulfur	Glutathione
	$nmol \cdot mg^{-1} \cdot min^{-1}$		nmol/mg protein	
Astrocytes	760 ± 122	217 ± 42	177 ± 33	12 ± 3
U373 cells	164 ± 20*	70 ± 12*	147 ± 23*	13 ± 2

* $p < 0.001$ (Student's *t*-test); if comparing U373 cells with astrocytes; each value is the mean of 15 to 112 repeats

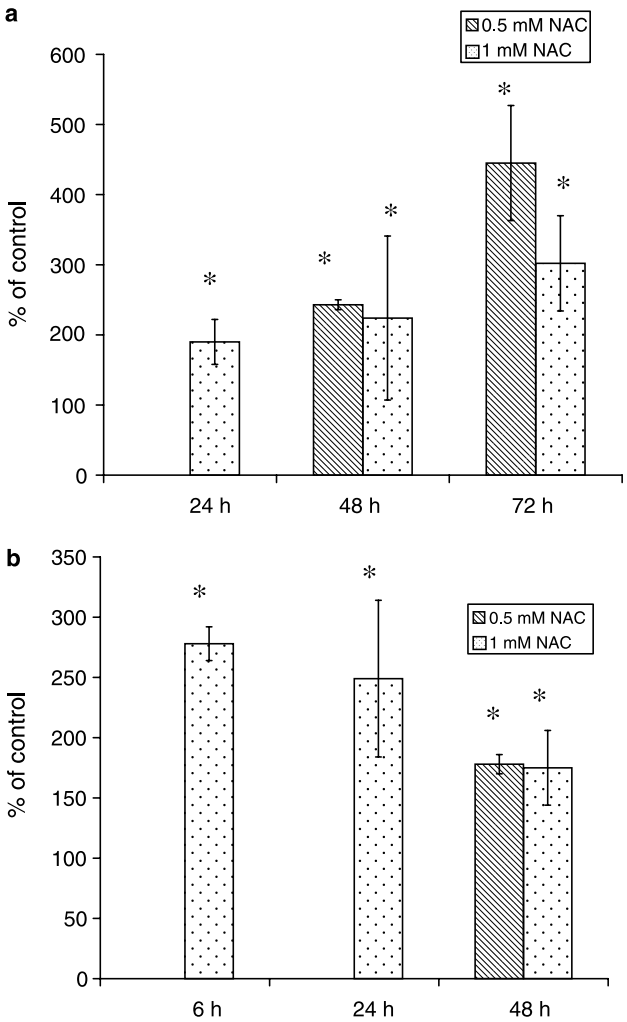


Fig. 1. The effect of NAC on glutathione levels in astrocytes (**a**) and U373 cells (**b**). *$P < 0.05$ (Student's *t*-test) vs. the controls. Each point represents mean ± SEM of 3-culture dishes from three different seedings. The control values are given in Table 1. The viability of the cells after each time point studied was between 98 and 100%

The cells cultured for 6, 12, 24, 48, and 72 h in the presence of 0.5 and 1 mM NAC showed an increased level of glutathione (Fig. 1a, b). In the U373 cells, the glutathione levels were from about 1.5 to about 3 times higher after 6, 24, and 48 h incubation with 1 mM NAC, and in astrocytes they were 2–4.5 times higher after 24, 48, and 72 h incubation.

The NAC effects on rhodanese and MPST activities and sulfane sulfur levels depended on its concentration in the culture medium and on the time of incubation. In mouse astrocytes, 1 mM NAC after 72 h of incubation, as compared with the controls, exerted no effect upon rhodanese activity, but the level of sulfane sulfur was significantly decreased and correlated with the decreased MPST activity (Fig. 2). These effects, together with significantly higher

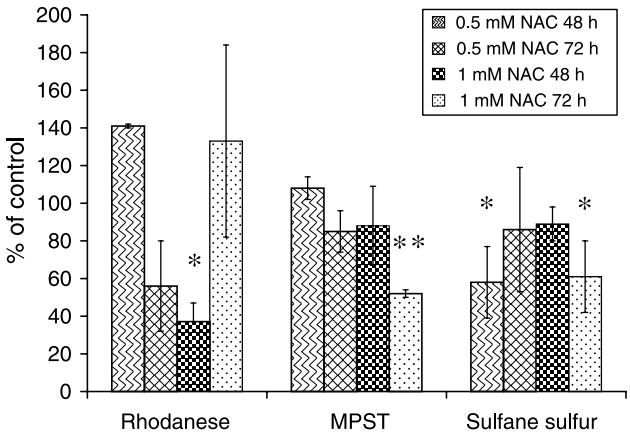


Fig. 2. NAC effect on rhodanese and MPST activities and sulfane sulfur levels in mouse astrocytes. $^{*}p<0.05$, $^{**}p<0.001$ (Student's *t*-test) vs. the controls. Each point represents mean ± SEM of 3-culture dishes from three different seedings. The control values are given in Table 1. The viability of the cells after each time point studied was between 98 and 100%

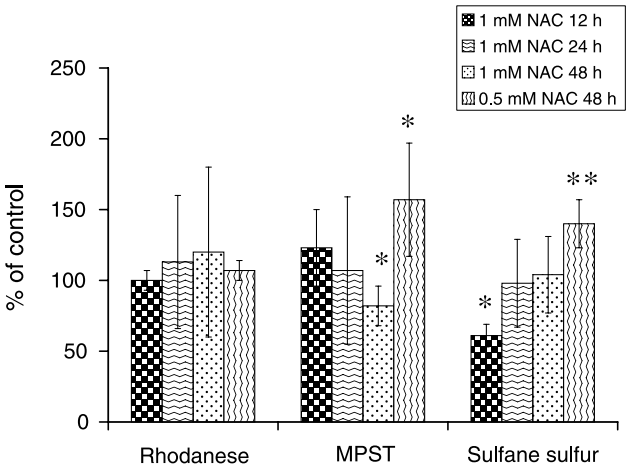


Fig. 4. NAC effect on rhodanese and MPST activities and sulfane sulfur levels in U373 cells. $^{*}p<0.05$, $^{**}p<0.001$ (Student's *t*-test) indicates means that were significantly different from the mean control value. Results are expressed as percentage of paired control values and were derived from triplicate assays performed on at least three culture groups of cells; error bars are S.E.M.

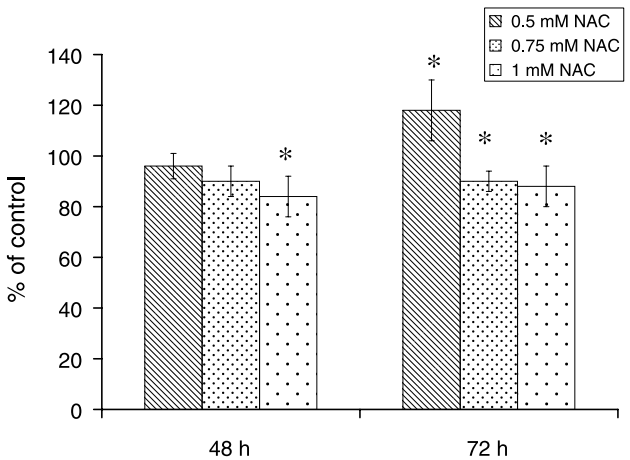


Fig. 3. NAC effect on mouse astrocytes' proliferation. $^{*}p<0.05$ (Student's *t*-test) vs. the control. Each point represents mean ± SEM of 3-culture dishes from three different seedings. The control values are given in Table 1. The viability of the cells after each time point studied was between 98 and 100%

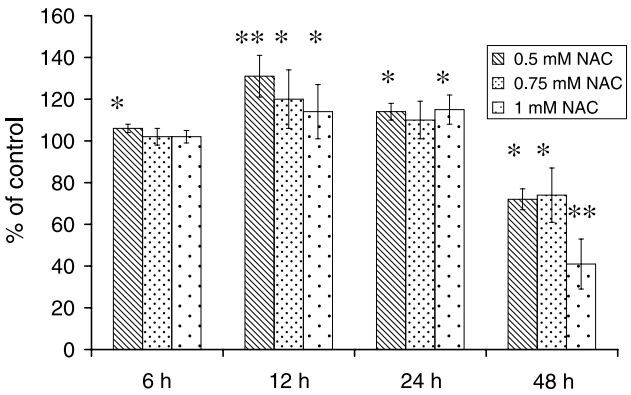


Fig. 5. NAC effect on U 373 cells' proliferation. $^{*}p<0.05$, $^{**}p<0.001$ (Student's *t*-test) indicates means that were significantly different from the mean control value. Results are expressed as a percentage of paired control values and were derived from triplicate assays performed on at least three culture groups of cells; error bars are S.E.M.

glutathione levels (Fig. 1a), accompanied decreased cell proliferation (Fig. 3). Diminished cell proliferation was also observed after 48 h of incubation with 1 mM NAC (Fig. 3), where rhodanese activity was decreased (Fig. 2), while the activity of MPST and the level of sulfane sulfur remained unchanged in comparison with the control cells; however, the glutathione level was elevated (Fig. 1a). 0.5 mM NAC after 72 h of incubation manifested no effect upon the activity of rhodanese and MPST and sulfane sulfur levels (Fig. 3), with the exception of GSH levels that were about 5 times higher (Fig. 1a) as compared with the controls, that stimulated cell proliferation (Fig. 3).

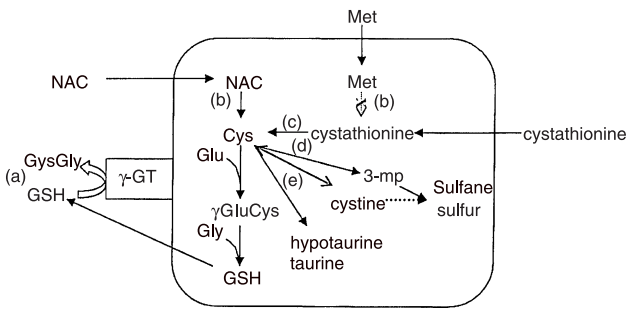
The same effect of increased cell proliferation in response to an elevated level of glutathione was observed in the human astrocytoma U373 cell line. 1 mM NAC after 24 h of incubation, as compared with the controls, did not exert any effect upon the rhodanese and MPST activities and sulfane sulfur levels (Fig. 4), with the exception of significantly higher GSH levels (Fig. 1b), which increased cell proliferation (Fig. 5). Diminished U373 cell proliferation was observed (1 mM NAC, 48 h) (Fig. 5) in the case where the MPST activity was lower in comparison with the controls, but the rhodanese activity and sulfane sulfur levels were not changed (Fig. 4), or the in-

creased MPST activity was accompanied by an increased level of sulfane sulfur (0.5 mM NAC, 48 h) (Fig. 4), which may suggest that U373 cell proliferation depended on the MPST activity and sulfane sulfur level. What is more, a decreased sulfane sulfur level (1 mM NAC, 12 h) (Fig. 4), together with stable MPST and rhodanese activity (Fig. 5) and an increased glutathione level (Fig. 1b), caused an increase in cell proliferation (Fig. 5).

Discussion

The presented investigations showed the basic differences in the intensity of L-cysteine desulfuration in murine astrocytes and the astrocytoma U373 line. It was found that in the U373 cells, as compared with astrocytes, the activities of MPST and rhodanese are significantly diminished and the level of sulfane sulfur is also lower (Table 1). In both types of brain cells, no γ-cystathionase activity was detected and it can be suggested that both the pathway of cysteine formation from methionine, and the pathway of sulfane sulfur formation from cystine through the γ-cystathionase reaction, do not play a significant role in the investigated cells (Schemes 1 and 2, dotted lines).

The antioxidant glutathione is essential for the detoxification of reactive radical species in brain cells (Cooper and Kristal, 1997; Dringen, 2000). Besides its intracellular function, glutathione also fulfils important extracellular functions in the brain. In this respect, astrocytes appear to play a key role in the glutathione metabolism of the brain, since astroglial glutathione is essential for providing glutathione precursors to neurons (Dringen and Hirrlinger, 2003). For many cell types in culture, it was demonstrated that cysteine in the culture medium limits maximal glutathione synthesis (Dringen and Hamprecht, 1996; Kranich et al., 1998). Our results have confirmed



Scheme 2. L-cysteine generation and transformation in astroglia cells. Based on: (a) Dringen et al. (1999), (b) Dringen and Hamprecht (1999), (c) Kranich et al. (1996), (e) Brand et al. (1988). 3-mp (3-mercaptopyruvate)

that astroglia cells and the U373 cells in culture are able to utilize NAC effectively as a cysteine donor for glutathione synthesis (Fig. 1). The increased glutathione level stimulates cell proliferation, which was observed for 0.5 mM NAC after 72 h in astrocytes culture (Figs. 1a and 3) or for 1 mM NAC after 24 h in astrocytoma culture (Figs. 1b and 5).

In this work, we investigated the effect of N-acetyl-L-cysteine on the MPST activity in neoplastic brain cells in order to determine their usability in stimulating cellular formation of sulfane sulfur and inhibiting cell proliferation. Such an effect was observed for 0.5 mM NAC after 48 h of incubation (Fig. 5). However, a diminished cellular proliferation was also detected for a higher, 1 mM NAC concentration in the culture medium (Fig. 5), at the same incubation period. It seems that a higher NAC or cysteine concentration in the cells exerts an inhibitory effect on the MPST activity. Under these conditions, no changes in the level of sulfane sulfur were detected, which may suggest not only that sulfane sulfur atoms may be important for some steps of the process of cell proliferation, but that the sulfurtransferase activity may be important in this respect also. On the other hand, a decreased sulfane sulfur level accompanied increased cell proliferation, as was found for 1 mM NAC after 12 h of incubation (Fig. 5). The results presented in this paper confirm that there is dependence between sulfane sulfur levels and the U373 cell proliferation – a lower level may contribute to an increased cell proliferation. NAC acts as a factor elevating sulfane sulfur level and modulating cell proliferation, but the effect depends on the duration. For 1 mM NAC (Fig. 4), the authors observed first a decrease in the sulfane sulfur level after 12 h and then, after 24 and 48 h, this level gradually increased. It is possible that cysteine released from NAC stimulates glutathione synthesis in cells and most of it is driven in this direction until the glutathione level reaches high enough values and the synthesis rate drops. The excess of cysteine is then used for other pathways, such as sulfane sulfur generation via the MPST reaction (Schemes 1 and 2). It is interesting that the activity of rhodanese in the U373 cells is stable and independent of NAC concentration and its time course. This may suggest that rhodanese is not sensitive to intracellular NAC and/or cysteine concentration.

We compared the effect of 1 mM NAC in the U373 cells and in mouse astrocytes. The most important observation was that in astrocytes the dependence between the sulfane sulfur level and cell proliferation is differ-

ent from that noted in the U373 cells. After 72 h, for 1 mM NAC, we observed a decreased cell proliferation (Fig. 3) accompanied by a decreased MPST activity correlated with a decreased sulfane sulfur level (Fig. 2). What is more, after 48 h, for 1 mM NAC, when the level of sulfane sulfur and the activity of MPST were unchanged, proliferation was also diminished, which was accompanied by a significantly decreased rhodanese activity (Figs. 2 and 3).

The availability of L-cysteine, which is also a glutathione precursor, may limit and modulate astroglial glutathione synthesis (Dringen et al., 1999). In neoplastic cells, the stimulation of cellular proliferation by an increased level of glutathione, as has been found for the U373 cells (1 mM NAC, 24 h), is harmful. These results seem to confirm the hypothesis postulated by Yildiz (2004), who suggested an inhibition of tumor growth by replacing glutathione (inhibition of its synthesis) with N-acetylcysteine. Glutathione depletion causes the inhibition of cell growth and enhances apoptosis; the deleterious effects of glutathione depletion may be overcome by utilization of NAC. NAC, like glutathione, can function as a soluble antioxidant (Dekhuijzen, 2004). Cysteine released from NAC can be driven to sulfane sulfur production (Schemes 1 and 2) and its increased level can in turn inhibit cell proliferation. One can hypothesize that a similar effect can be also reached with cystathionine, which also has an antioxidative property and may serve as a cysteine precursor (Kranich et al., 1996). However, under such conditions, γ-cystathionase activity, which is necessary to convert cystathionine to cystine (Scheme 2), has to be activated at the protein level or induced at the level of gene expression, which should be verified in the future.

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