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Cysteamine Increases Homocysteine Export and Glutathione Content by Independent Mechanisms in C3H/10T1/2 Cells

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SUMMARY

Several thiols, including homocysteine and cysteamine, have been shown to increase glutathione levels in C3H/10T1/2 Cl 8 cells [*Biochem. Pharmacol.* 39:421–429 (1990)]. The present paper shows that cysteamine also increases homocysteine export from these cells. Cellular glutathione content and export of glutathione and homocysteine increased with increasing doses of cysteamine. Twenty-four hours after addition, 300 μM cysteamine increased both glutathione content and homocysteine export 3–4-fold. No change in the ratio between reduced and oxidized glutathione could be detected, suggesting that the cysteamine effect was not due to reduction of pools of oxidized glutathione. The elevation of glutathione occurred rapidly but declined between 24 and 48 hr after addition of cysteamine, whereas the homocysteine export increased momentarily after cysteamine exposure and then proceeded at a rate similar to that from untreated control cells. The cysteamine-induced increase in glutathione was completely prevented by the γ-glutamylcysteine synthetase inhibitor buthionine sulfoximine but was not affected by inhibition of homocysteine formation by 3-deazaaristeromycin. Buthionine sulfoximine did not prevent the increase in homocysteine export by cysteamine, and only a small increase in homocysteine export was observed when the cells were exposed to 3-deazaaristeromycin before treatment with cysteamine. Two major conclusions were drawn. 1) Increase of glutathione content and homocysteine export by cysteamine were independent events, indicating that glutathione status and homocysteine formation are regulated by independent mechanisms in C3H/10T1/2 Cl 8 cells. 2) *S*-Adenosylhomocysteine catabolism was the main source of the homocysteine export induced by cysteamine.

Hcy is sulfur-containing amino acid not found in the diet and is a metabolic precursor for both methionine and cystathione synthesis. The only known source of Hcy in vertebrates is the catabolism of AdoHcy, and Hcy is further metabolized to methionine through remethylation or is converted to cystathionine and cysteine via the transsulfuration pathway. The intracellular level of Hcy seems to be strictly regulated by export mechanisms (1). Under normal conditions cells excrete considerable amounts of Hcy, but perturbation of metabolism leading to accumulation of Hcy results in enhanced export of the amino acid (2). The enzyme responsible for catabolism of AdoHcy has been the target for cytostatic action of numerous purine analogues, including c^3Ari^1 and 3′-deazaadenosine (3,4). Inhibition of the formation of Hcy by such compounds is followed by a drastic decrease in Hcy export (5, 6), whereas an increased endogenous supply of methionine leads to a similar increase in Hcy export (7). The intracellular formation of Hcy thus seems to be reflected in the export of the compound. It should be noted that Hcy in the extracellular medium itself may be cytotoxic and may represent a pathogenic factor provoking arterial lesions (1, 8), making Hcy export an important area of investigation.

Because Hcy is a precursor of cystathionine and subsequently cysteine, which is a substrate of the first step in the synthesis of GSH (9), a relation between Hcy formation and GSH synthesis might exist. GSH is the most abundant cellular nonprotein thiol (9, 10), and changes in GSH level may affect the distribution of other thiols, including Hcy, between the reduced and oxidized forms. According to several reports, cysteine and intracellular cysteine-generating agents increase GSH level (10), and thiols that are not part of the cystathionine/GSH pathway have also been shown to increase GSH (11), apparently by promoting cystine uptake. In addition, it has been demonstrated that at least some cell types utilize cysteine supplied by the transsulfuration pathway for GSH synthesis (12).

We have recently shown that Hcy (13) and several other thiols (14) increased glutathione levels in nontransformed C3H/10T1/2 Cl 8 cells. Cysteamine was most efficient, increasing the GSH content 5-fold above control level in confluent cells (14). Furthermore, the increase in GSH level was accom-

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ABBREVIATIONS: Hcy, homocysteine (form not specified); BSO, L-buthionine-(*S*,*R*)-sulfoximine; c^3Ari, 3-deazaaristeromycin; GSH, reduced glutathione; GSSG, oxidized glutathione; GSSR, soluble glutathione mixed disulfides; AdoHcy, *S*-adenosylhomocysteine.

panied by enhanced export of Hcy, which pointed to the possibility of a link between Hcy formation and GSH synthesis.

However, depleting of exponentially growing C3H/10T1/2 Cl 8 cells of glutathione by BSO, a well known irreversible inhibitor of the first step in GSH synthesis (15, 16), did not support this view, because no significant effect on the Hcy export rate was observed (14).

Agents modulating GSH level are becoming increasingly important in cancer chemotherapy (10, 17, 18). GSH partly protects the cells against the action of several cytostatic drugs (10, 17), thus reducing the cytostatic effect of these agents. Conversely, some drugs require chemical reduction before exerting their cytostatic effect (17); this can be achieved by increasing the intracellular level of GSH. Modulation of GSH level, thus, may play an important part in future cancer chemotherapy, and BSO, which efficiently depletes cells of glutathione, is already being introduced into clinical trials (17, 18).

With this background, studies concerning the effects of GSH-modulating agents on metabolic pathways are greatly required. In the present paper, we report detailed studies on the effect of cysteamine on GSH content and Hcy export from cells in culture.

The question of a possible link between Hcy formation and GSH synthesis was approached by a two-step experimental design. First, GSH synthesis was inhibited by BSO and the effect of cysteamine on Hcy export and GSH level was observed. Second, Hcy formation was blocked by c^3Ari, a competitive inhibitor of AdoHcy hydrolase (19), and the effect of cysteamine on Hcy export and GSH level was determined. Our data strongly suggest that cellular GSH content and formation of Hcy are mediated by independent mechanisms.

Materials and Methods

Chemicals

Cysteamine, dithioerythritol, AdoHcy, BSO, GSH, and GSSG were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium borohydride was from Fluka Chemie AG (Buchs, Switzerland) and monobromobimane was from Calbiochem, Behring Diagnostics (La Jolla, CA). c^3Ari was kindly supplied by Dr. John A. Montgomery, Southern Research Institute (Birmingham, AL).

Cell Line and Culture Conditions

Stock cultures of nontransformed C3H/10T1/2 Cl 8 mouse embryo fibroblasts (20) were maintained as described previously (8). In all experiments cells were seeded in plastic dishes (6 cm; Nunc, Roskilde, Denmark) in Basal Medium Eagle (Flow Laboratories, Herts, UK) supplemented with 10% heat-inactivated fetal calf serum (Sera-Lab, Ltd., Sussex, UK) and were grown at 37° in an atmosphere of 5% CO_2 in air and a relative humidity of 95%.

One to two days after the cells had reached confluence, the experimental protocol was initiated by replacement of the medium with fresh medium containing cysteamine at the concentrations indicated. In some experiments the cells were pretreated by replacement of the medium with fresh medium containing either BSO (20 μM) or c^3Ari (50 μM) and, 8 hr after addition of BSO or 1 hr after addition of c^3Ari, cysteamine (100 μM) was added directly to the medium. At different times after initiation of treatment, the cells were harvested for determination of glutathione by removal of the culture medium, gently washed twice with ice-cold phosphate-buffered saline, and immediately frozen at −85°. Samples of the medium were frozen at −85° for determination of extracellular Hcy and glutathione.

For determination of cell number at harvesting, two parallel dishes from each group were removed and the cells were trypsinized and counted using a Coulter Counter Model ZM (Coulter Electronics Ltd., Luton, UK).

Determination of Glutathione

Intracellular levels. The frozen cells (−85°) were extracted with ice-cold 5% sulfosalicylic acid and scraped off the dish with a rubber policeman, and the precipitated proteins were removed by centrifugation.

Extracellular levels. Ice-cold sulfosalicylic acid was added to the medium (5% final concentration) and the precipitated proteins were removed by centrifugation.

GSH was determined in the acid supernatants by derivatization with monobromobimane (Kosower's reagent) and subsequent quantitation of the GSH-bimane derivative by high pressure liquid chromatography as previously described (13). After reduction with sodium borohydride, the same procedure was used to determine total soluble glutathione (GSH plus GSSG plus GSSR).

Determination of Extracellular Hcy

The medium was mixed with perchloric acid to remove proteins, neutralized, and treated with dextran-coated charcoal to remove nucleosides and AdoHcy, which would interfere with the Hcy assay (5, 6). Hcy was then determined as described previously (21).

Determination of Protein

Protein was determined according to the method of Bradford (22), using the Bio-Rad protein assay kit. Bovine γ-globulin was used as protein standard.

Results

Effect of cysteamine concentration on glutathione content and Hcy export. Cysteamine at concentrations ranging from 10 to 300 μM increased cellular glutathione content in a dose-dependent manner (Fig. 1). Cysteamine at 300 μM resulted in a 3.6-fold increase in glutathione 24 hr after addition. Both reduced and total glutathione were determined, and GSH accounted for most cellular glutathione (Fig. 1). Cysteamine did not significantly change the GSH/GSSG ratio.

When the cells were exposed to various concentrations of cysteamine, there was also a dose-dependent increase in extracellular glutathione (determined as total glutathione). Furthermore, a similar dose-dependent effect was observed on the export of Hcy. Cysteamine at 300 μM resulted in a 3.4-fold increase in Hcy export 24 hr after addition (Fig. 1).

Time course for the effect of cysteamine on glutathione content and Hcy export. Cysteamine elevated the intracellular glutathione content very rapidly; 4 hr after addition of 100 μM cysteamine, the GSH content was increased 4-fold (Fig. 2A). During the time course, GSH accounted for most cellular glutathione, and again no significant change in the GSH/GSSG ratio could be observed.

The export of Hcy was instantaneously increased by addition of 100 μM cysteamine, but 4–5 hr later the export rate seemed to be nearly constant and not significantly different from the control (Fig. 2B).

Effect of cysteamine on glutathione-depleted cells. The possibility that cysteamine acted by releasing glutathione from pools containing protein-glutathione mixed disulfides was investigated by inhibiting γ-glutamylcysteine synthetase by BSO. We have recently shown that 20 μM BSO reduces the GSH content of these cells to 5.5% of control within 24 hr, with no or minimal cytotoxic effects (14). The cells were, therefore, treated with 20 μM BSO for 8 hr and then exposed to 100 μM

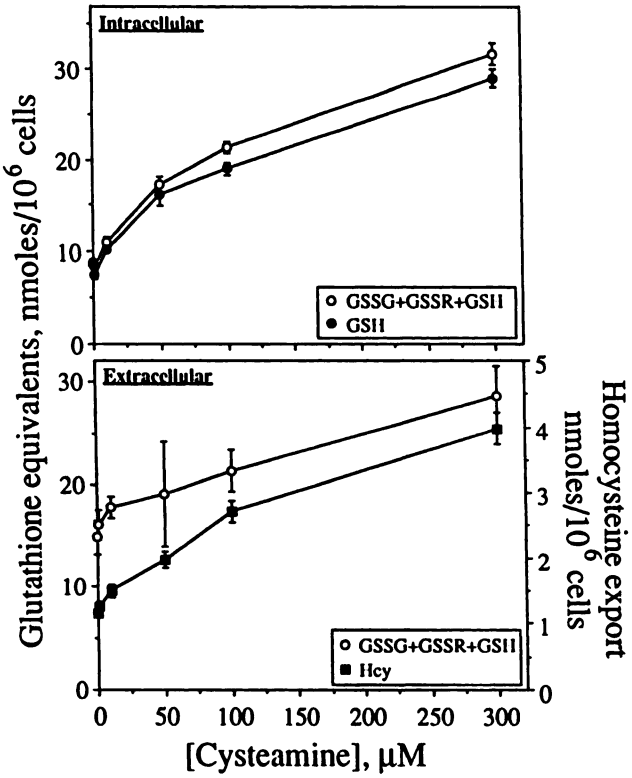

Fig. 1. The effect of cysteamine concentration on glutathione content and Hcy export. One day after confluence was reached the medium was replaced by fresh medium containing different concentrations of cysteamine. Twenty-four hours later, the intracellular amount of glutathione and the amounts of Hcy and glutathione in the medium (extracellular) were determined. The results are expressed as equivalents of GSH or Hcy and represent the average of four determinations ± standard deviation.

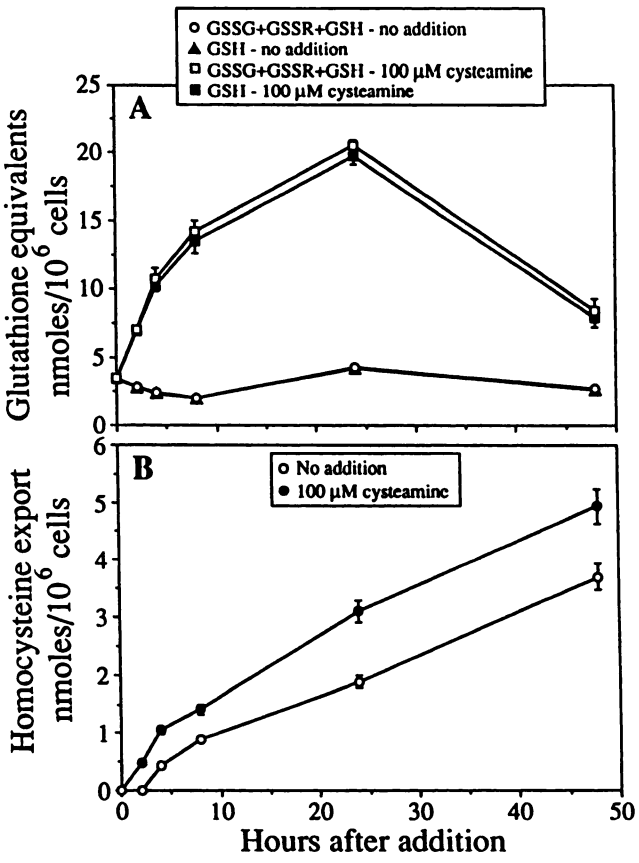

Fig. 2. Time course for the effect of cysteamine on glutathione content and Hcy export from confluent C3H/10T1/2 Cl 8 cells. Two days after confluence was reached, the medium was replaced by fresh medium containing either no addition or 100 μM cysteamine. At the times indicated, the intracellular content of glutathione (A) and the amount of Hcy (B) in the medium were determined. The results are expressed as equivalents of GSH or Hcy and represent the average of four determinations ± standard deviation.

cysteamine. As seen in Fig. 3A, BSO completely prevented the increase in glutathione by cysteamine, indicating that the elevation of glutathione was solely due to increased GSH synthesis.

This experimental protocol was further utilized to investigate whether the increased Hcy export observed after cysteamine exposure (Figs. 1 and 2A) was due to the elevated GSH level. The data presented in Fig. 3B showed that 20 μM BSO had no significant effect on the Hcy export. Furthermore, the results demonstrated that cysteamine increased the Hcy export in BSO-treated cells, at least to the same extent as in the untreated control cells.

Effect of cysteamine on Hcy-depleted cells. The next question addressed was whether the increased Hcy export (Figs. 1, 2B, and 3B) was the result of an increased catabolism of AdoHcy, which at present is the only known source of Hcy, or was due to release of protein-bound or compartmentalized Hcy. Therefore, the cells were treated with c^3Ari, which is a well known competitive AdoHcy hydrolase inhibitor (19) that has been shown to block Hcy formation almost instantaneously (5). The results demonstrated that c^3Ari almost completely prevented the Hcy export (Fig. 4B). Addition of cysteamine 1 hr after c^3Ari induced only a small increase in Hcy export, indicating that AdoHcy was the major source of the increased Hcy formation induced by cysteamine.

Blocking of the Hcy formation had no effect on the glutathione content (Fig. 4A), supporting the findings described above, which indicated that Hcy formation and GSH synthesis are regulated by independent mechanisms.

Discussion

In our previous report (14), we studied the effect of several thiols, including Hcy and cysteamine, on glutathione content in C3H/10T1/2 Cl 8 cells and showed that among the thiols tested cysteamine was the most efficient in raising the glutathione level. By depleting the cellular glutathione content with BSO, we showed that the Hcy export was not affected, indicating that decreased glutathione levels did not influence a normal formation rate of Hcy.

In the present work, the modulating effect on cellular glutathione content and Hcy export by cysteamine was studied in detail. The results showed that the rise in glutathione content was dose dependent and increased with increasing concentrations of cysteamine (Fig. 1). Most of the glutathione was in the reduced form, and both the total and the reduced glutathione increased to approximately the same extent when the cells were treated with cysteamine (Figs. 1 and 2A). This is in agreement with recent reports (11, 23) and indicated that cysteamine did not act by mobilizing glutathione from pools of oxidized glutathione, but rather by stimulating GSH synthesis.

Notably, cysteamine had a significant effect at concentra-

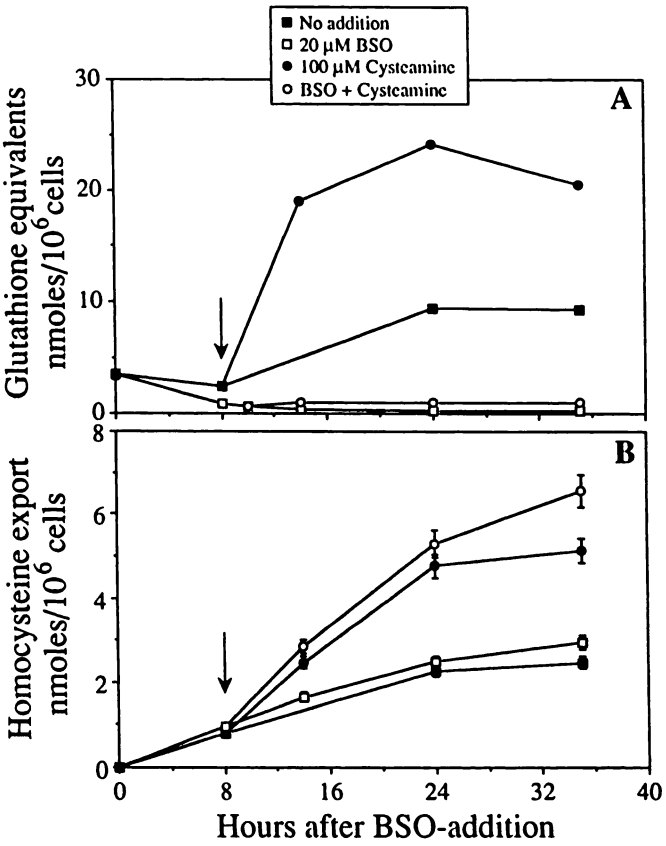

Fig. 3. Effect of cysteamine on glutathione content and Hcy export in glutathione-depleted C3H/10T1/2 Cl 8 cells. One day after confluence was reached, the medium was replaced by fresh medium containing either no addition or 20 μM BSO. Eight hours later, 100 μM cysteamine was added directly to the dishes as indicated (*arrows*), and the intracellular content of glutathione (A) and the amount of Hcy (B) in the medium were determined at different times thereafter. The results are expressed as equivalents of GSH or Hcy and represent the average of four determinations ± standard deviation.

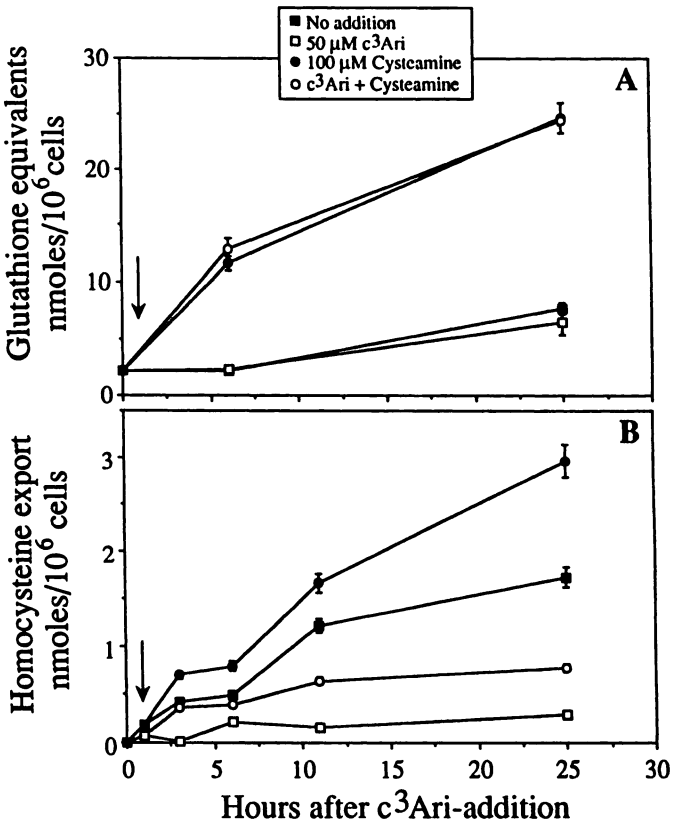

Fig. 4. Effect of cysteamine on glutathione content and Hcy export in Hcy-depleted C3H/10T1/2 Cl 8 cells. One day after confluence was reached, the medium was replaced by fresh medium containing either no addition or 50 μM c^3Ari. One hour later, 100 μM cysteamine was added directly to the dishes as indicated (*arrows*), and the intracellular content of glutathione (A) and the amount of Hcy (B) in the medium were determined at different times thereafter. The results are expressed as equivalents of GSH or Hcy and represent the average of four determinations ± standard deviation.

tions as low as 10 μM, increasing cellular glutathione by 24%, whereas 50 μM resulted in a 94% increase in cellular glutathione content (Fig. 1). These results show that cysteamine was effective also at concentrations within the range between 35 and 60 μM, obtained in plasma after oral administration of cysteamine (24).

Furthermore, a similar dose-dependent increase was observed in the export of both glutathione and Hcy (Fig. 1). These results are consistent with previous findings that cells excrete glutathione at rates proportional to the intracellular level (9, 18, 25) and may, thus, indicate that the intracellular glutathione level is partly regulated by export mechanisms. The increased export of Hcy induced by cysteamine and the simultaneous increase in both content and export of glutathione (Fig. 1) might suggest a link between Hcy formation and glutathione status.

Our results showed that the export of Hcy was momentarily increased by cysteamine, but then the export rate became nearly constant and equal to that of the control (Fig. 2B). The glutathione level also increased very rapidly but, in contrast to the Hcy export, it reached a high level and then declined between 24 and 48 hr after addition of cysteamine (Fig. 2). This finding does not support the suggestion of glutathione level controlling Hcy formation or export.

The succeeding experiments (Fig. 3) then answered two important questions. First, it has been well documented that several pools/compartments of glutathione exist in the cells (26), of which the mitochondrial pool is the most extensively studied next to the cytosolic GSH (27, 28). Because we are disrupting all cellular compartments during the sulfosalicylic acid extraction and, thus, are measuring the total cellular glutathione content, the observed increase after cysteamine exposure could not be due to leakage or transport of glutathione from mitochondria or other cellular pools to the cytosol. However, we could not exclude the possibility that glutathione existed in a form bound to macromolecules that were precipitated and removed during the acid extraction and that this bound form was released as free glutathione by the action of cysteamine. Notably, the increase in glutathione was completely prevented by BSO (Fig. 3A), showing that the increased GSH content derived from new synthesis of GSH and not from release of GSH bound to any macromolecular structures. Similar results have been demonstrated for the effect of cysteamine and *N*-acetylcysteine on glutathione content in Chinese hamster cells (11).

Second, the present results (Fig. 3B) confirmed our recent findings that depletion of glutathione in C3H/10T1/2 Cl 8 cells had no effect on the export of Hcy (14). Furthermore, cysteamine increased the Hcy export in glutathione-depleted cells, at

least to the same extent as in untreated control cells, strongly indicating that the modulation of GSH and Hcy by cysteamine were independent events. Further evidence for this view were the results showing that inhibition of Hcy formation by c^{3}Ari did not affect the ability of cysteamine to increase cellular glutathione (Fig. 4A).

A similar approach was chosen to investigate the origin of the increased Hcy export. Because the only known source of Hcy is the catabolism of AdoHcy (1, 29), inhibition of the enzyme responsible for the degradation should prevent the increase in Hcy export. The results gave no evidence for any unknown source of Hcy, although a small initial increase in Hcy export compared with control was observed (Fig. 4B). This may be due to residual enzyme activity, which has been reported for several nucleoside analogues inhibiting AdoHcy hydrolase (30, 31). A small residual egress of Hcy after exposure of cells to c^{3}Ari at concentrations up to 300 μM has also been demonstrated (5, 13).

A possible hypothesis for the observed effects of cysteamine can be suggested from the investigations into the disease cystinosis, which is characterized by an inherited error of cystine metabolism, leading to accumulation of cystine within lysosomes and subsequent renal failure (32). Several aminothiols including cysteamine, dithiothreitol, and pantetheine have been shown to lower cystine content in cultured fibroblasts derived from cystinosis patients and in isolated lysosomes (32–34). Cysteamine and pantetheine have proved to be most efficient in this respect, and patients suffering from this disease are currently treated with cysteamine. The proposed mechanism behind the therapeutic effect is as follows. Cysteamine enters the lysosomes, reacts with cystine, and forms cysteine-cysteamine mixed disulfide and cysteine, both of which leave the lysosomes (32). The mixed disulfide is further transported into the extracellular space (34), whereas cysteine is either transported out of the cell or used for intracellular synthesis of GSH (32, 33). Due to the close structural resemblance of cysteine and Hcy, it is conceivable that a cysteamine-Hcy mixed disulfide also is formed and exported out of the cell. This may provide a possible explanation for the observed phenomena in our experiments and the apparent independence of the two events. Cysteamine may facilitate transport of Hcy out of the cell as cysteamine-Hcy mixed disulfide, while cysteamine simultaneously enters the lysosomes, facilitates transport of cysteine out of the lysosomes, and then enters the GSH synthesis. The net result of these processes is then enhanced intracellular glutathione content and increased Hcy egress. Furthermore, these events will occur independently of each other, because blocking of GSH synthesis is not likely to affect the transport of the cysteamine-Hcy disulfide and, similarly, blocking of the catabolism of AdoHcy would not affect the export of the cysteamine-cysteine mixed disulfide from the lysosomes and the increased availability of cysteine for GSH synthesis.

In conclusion, the present data show that cysteamine enhances both intracellular glutathione content and Hcy formation in nontransformed C3H/10T1/2 Cl 8 cells and that these effects increase with increasing doses of cysteamine. The increase in glutathione is probably due to new synthesis of GSH, and AdoHcy catabolism is shown to be the main source of the increased Hcy formation. Our results show that the increase in glutathione content and Hcy export by cysteamine are independent events, suggesting that Hcy formation and glutathione status are regulated by independent mechanisms. A hypothesis explaining the biochemical basis of these effects is proposed and should be further evaluated.

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