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Role of glutathione in the accumulation of anticarcinogenic isothiocyanates and their glutathione conjugates by murine hepatoma cells

Yuesheng Zhang

Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

Email: yzhang@jhmi.edu

Isothiocyanates (ITCs) are abundant in the human diet. Many potently inhibit tumorigenesis induced by a wide variety of chemical carcinogens in rodents. Recently, we observed that several ITCs accumulated to very high concentrations in cultured cells and that their accumulated levels were closely related to their potencies in inducing phase II enzymes [NAD(P)H:quinone reductase and glutathione transferases] that detoxify carcinogens. To elucidate the molecular mechanism responsible for this accumulation, the intracellular chemical identities of two ITCs, sulforaphane [SF, 1-isothiocyanato-(4*R*,*S*)-(methylsulfinyl)butane] and benzyl-ITC, were investigated in murine hepatoma cells. Both ITCs accumulated very rapidly to high intracellular concentrations, but, remarkably, most of the intracellular forms of the ITCs were dithiocarbamates resulting from conjugation with reduced glutathione (GSH). For example, the intracellular concentration reached 6.4 mM when cells were exposed to 100 μM SF for 30 min at 37°C and 95% of the accumulated product was the GSH conjugate. Cellular accumulation of each ITC was accompanied by a profound reduction in cellular GSH levels. These findings, together with our previous observation that accumulation of ITCs depended on cellular GSH levels, strongly suggest that intracellular conjugation of ITCs with GSH is mainly responsible for ITC accumulation. Surprisingly, rapid accumulation to high concentrations also occurred when cells were exposed to the GSH–ITC conjugates. However, these conjugates were apparently not absorbed intact, but were hydrolyzed extracellularly to free ITCs that were taken up by the cells. This conclusion is supported by the finding that suppression of dissociation of the conjugates by excess GSH or other thiols blocks accumulation of the conjugates.

Introduction

Many isothiocyanates (ITCs) prevent chemical carcinogenesis in animals by mechanisms that are believed to include enhancing detoxication of reactive carcinogens by inducing phase II enzymes, blocking carcinogen activation by inhibiting certain phase I enzymes and stimulating apoptosis of damaged cells (1–4). ITCs and their glucosinolate precursors are widely and sometimes abundantly distributed in vegetables, especially in crucifers (5). Individual daily consumption of total ITCs in humans may range from a few to several hundred micromoles (6–9) and it is therefore possible that ITCs may be partly responsible for the epidemiological observations that high consumption of vegetables, and especially of crucifers, by humans is closely correlated with a reduced risk of developing many types of cancer (10,11). There are large differences in the biological potencies among ITCs both *in vivo* and *in vitro*, and the mechanisms underlying these differences remain unclear (3,12,13).

The studies presented in this paper were prompted by our recent finding that several ITCs accumulated rapidly to very high concentrations in several cell lines, that the degree of such accumulation was closely correlated with their potencies in inducing phase II detoxification enzymes (glutathione transferases and quinone reductase) in murine hepatoma Hepa 1c1c7 cells and that those ITCs that did not accumulate were not inducers (14). We further showed that prior depletion of reduced glutathione (GSH) in Hepa 1c1c7 cells (by inhibiting GSH synthesis) significantly reduced the cellular accumulation of several ITCs, suggesting that conjugation of ITCs with GSH may be critical for their accumulation (14). Since induction of phase II enzymes is an important strategy for reducing susceptibility to carcinogens (15,16), these findings raised the possibility that accumulation of ITCs in cells may play a critical role in determining their anticarcinogenic activity.

This study was designed to determine the chemical identity of the ITCs that accumulate intracellularly and to determine, in particular, the role of glutathione in their accumulation. The present study focused on two ITCs, sulforaphane (SF) and benzyl isothiocyanate (benzyl-ITC) (see Figure 1 for chemical structures). These compounds were chosen because they are rapidly accumulated to very high concentrations in several cultured cell lines, are naturally abundant in edible plants and display potent chemoprotective activity in animals (14,17–20). Hepa 1c1c7 cells were selected as a model system because most of our earlier work was done with this line and other cells behave similarly with respect to their uptake kinetics for ITCs.

Benzyl-ITC is metabolized via the mercapturic acid pathway in both humans and laboratory animals (21,22). Preliminary experiments have shown that SF is also largely metabolized by this pathway in humans (our unpublished data), which requires that ITCs first be conjugated with GSH to form the corresponding dithiocarbamates, which are then converted sequentially to conjugates with cysteinylglycine, cysteine and *N*-acetylcysteine (mercapturic acids). Whether the accumulated ITCs are conjugated with GSH and the relationship of these conjugates to ITC uptake and accumulation are the subjects of this report. Answers to these questions are critical for understanding the anticarcinogenicity as well as the toxicity of ITCs.

The work presented in this paper shows that SF is almost exclusively accumulated as the GSH conjugate in Hepa 1c1c7 cells, whereas benzyl-ITC is less completely but still predominantly accumulated as the GSH conjugate. Although the

Abbreviations: ARE, antioxidant-responsive elements; benzyl-ITC, benzyl isothiocyanate; BSO, buthionine-(*R*,*S*)-sulfoximine; GSH, reduced glutathione; ITC, isothiocyanate; SF, sulforaphane [1-isothiocyanato-(4*R*,*S*)-(methylsulfinyl)butane].

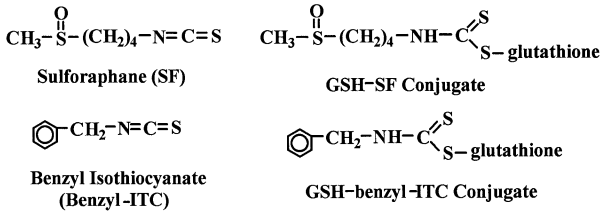

Fig. 1. Chemical structures of sulforaphane, benzyl isothiocyanate and their GSH conjugates (dithiocarbamates).

conjugates appeared to be short-lived in cells, they were not further metabolized by the mercapturic acid pathway in these cells. The rapid accumulation of ITCs profoundly depleted cellular GSH levels. Further experiments have shown that exposure of Hepa cells to GSH–ITC conjugates (see Figure 1 for chemical structures of the conjugates) in the medium also resulted in accumulation of the conjugates, but that hydrolysis of the extracellular conjugates was required for uptake. Information on the cellular uptake of conjugates is important since GSH and other thiol conjugates of some ITCs have been shown to inhibit phase I enzymes (23), to induce phase II enzymes (our unpublished data) and to inhibit tumorigenesis in animals (24,25).

Materials and methods

Chemicals

Synthetic SF was a gift from Professor G.H.Posner (Department of Chemistry, The Johns Hopkins University). Benzyl-ITC, 1,2-benzenedithiol, *N*-acetylcysteine, buthionine (*R*,*S*)-sulfoximine (BSO) and tetraethylammonium bromide were purchased from Aldrich (Milwaukee, WI). The first two compounds were distilled before use. Dibutyl phthalate, diisononyl phthalate, glutathione, L-cysteine and cysteinylglycine were purchased from Sigma Chemical Co. (St Louis, MO).

Preparation of ITC–thiol conjugates

Most ITCs react avidly with thiols under mild conditions to give rise to the corresponding dithiocarbamates (26). SF and benzyl-ITC was each allowed to react with twice the molar concentration of GSH, cysteinylglycine, cysteine or *N*-acetylcysteine in water at pH 8.5 (adjusted with KOH) for 1 h at room temperature. Each solution was then exhaustively extracted with ethyl acetate to remove the residual ITC and the aqueous phase was examined spectroscopically for presence of the conjugates, which were measured by the cyclocondensation assay which quantifies all conjugates (27). All thiol–ITC conjugates have similar UV absorbance spectra which are characteristic of dithiocarbamates and display absorption maxima at ~250 nm (a_m ~ 10 000/M/cm) and shoulders near 270 nm. The conjugates were prepared just before use and stored at 4°C to retard dissociation.

Cell culture

Hepa 1c1c7 cells were cultured in α-MEM plus 10% fetal bovine serum (treated with 1% charcoal at 55°C for 90 min and then filtered) (28) in 5% CO_2 at 37°C in a humidified incubator.

Measurement of intracellular accumulation of ITCs and their GSH conjugates

The procedures involving cell exposure to ITCs or their GSH conjugates and preparation of cell lysates were slightly modified from those described in a previous publication (14). Briefly, 7×10^5 cells were plated in a 10 cm plastic Petri dish with 10 ml of medium. After 3 days, the cell number reached $4–5\times10^6$ cells/plate. Cell monolayers were either directly exposed to a test compound or cell suspensions were prepared by treating the cells with 1 ml of 0.05% trypsin/plate for 2–4 min and then mixing with 9 ml of medium. Monolayers or cell suspensions in 10 ml volumes were exposed to the specified concentrations of the test compounds (dissolved in 5–50 µl of acetonitrile) in a 10 cm Petri dish at 37°C for the specified times. In experiments where GSH, cysteine or *N*-acetylcysteine was added to the medium, the pH of the medium was adjusted to its original value with sodium hydroxide. In experiments involving pretreatment with BSO, cell monolayers in 10 cm plates with 10 ml of medium were incubated with 100 µM BSO for 24 h, trypsinized and then suspended in medium as described above. The cell suspensions were exposed to the test compound in the presence of 100 µM BSO. For all experiments, at the end of the exposure the entire suspension (monolayer cells were trypsinized and suspended in medium) was quickly transferred to a 15 ml tube containing 2 ml of equal volumes of dibutyl phthalate and diisononyl phthalate and centrifuged at 3000 r.p.m. (Sorvall RT6000; Du Pont, Wilmington, DE) at 4°C for 2 min to separate the cells from the medium. The medium and oil were removed by aspiration and the tube tip containing the cell pellet was retrieved. The entire pellet was then resuspended in 200 µl of H_2O and stored at –70°C overnight. The cells were thawed and sonicated with a model 200 sonifier (Branson, Danbury, CT).

Cellular accumulation levels of ITCs and conjugates were determined by the cyclocondensation assay. This assay involves the quantitative condensation of ITCs and/or dithiocarbamates with an excess of 1,2-benzenedithiol under conditions that produce stoichiometric quantities of 1,3-benzodithiole-2-thione, which is then quantified spectroscopically (27,29). The method does not distinguish ITCs from dithiocarbamates and measurements of the accumulated material therefore include ITCs, their conjugates and potentially other ITC derivatives. The assay protocol and conversion of measured values for ITCs and conjugates in the lysates to intracellular concentrations were as reported (14). The terms 'conjugates' and 'intracellular ITCs' are used interchangably in this paper to designate free ITCs and the dithiocarbamates derived from them by condensation with molecules containing sulfhydryl groups, including GSH, cysteinylglycine, cysteine and *N*-acetylcysteine. All of these compounds are detected and quantified by the cyclocondensation assay. Therefore, unless otherwise specified, the quantities of accumulated compounds in cells or cell lysates reported in this paper refer to the total amounts of ITCs and their dithiocarbamate derivatives without specifying their chemical identities.

Separation of ITCs and their thiol conjugates by paired-ion HPLC

To determine the chemical identity of ITCs in cells, paired-ion HPLC separation procedures were developed to establish the retention times of each ITC and its thiol conjugates. GSH, cysteinylglycine, cysteine and *N*-acetylcysteine conjugates are the intermediates and final metabolites of mercapturic acid synthesis from ITCs (30). Although studies have indicated that cyclic mercaptopyruvic acids may also arise from ITCs in mice (31), these compounds were not detected in Hepa 1c1c7 murine hepatoma cells.

An analytical reverse phase column (Whatman Partisil 10 µm ODS-2, 4.5×250 mm) was eluted with solvents at a rate of 2 ml/min. Elution of each compound was monitored at 250 nm and its identity was confirmed by simultaneous scanning of the absorption spectrum with a photodiode array detector (Waters model 996) and quantified by cyclocondensation assay of each fraction. Two solvents were used in the gradient: solvent A, 6 mM tetraethylammonium bromide in water; solvent B, acetonitrile. For separation of SF and its four conjugates, two successive linear gradients were run: (i) from 93% solvent A and 7% solvent B to 85% solvent A and 15% solvent B for the first 7 min; (ii) from 85% solvent A and 15% solvent B to 20% solvent A and 80% solvent B over the next 10 min. For separation of benzyl-ITC and its four conjugates, the run for the first 9 min was isocratic with 82% solvent A and 18% solvent B, followed by a linear gradient to 100% solvent B over the next 3 min and was maintained at 100% solvent B thereafter. The retention times of each compound are shown in Figure 2. Although cysteine–benzyl-ITC and *N*-acetylcysteine–benzyl-ITC were not completely separated by HPLC, this is insignificant because neither compound was detected in cell lysates.

To elucidate the chemical identities of ITCs in cells, Hepa 1c1c7 cells were exposed to each ITC at specified concentrations and for specified times and the cells were then separated from the medium and lysed, as described above. The cell lysates were either applied directly to the paired-ion HPLC or were first passed through a Centricon filter at 4°C (hydrophilic membrane, 3000 mol. wt pore size; Amicon, Beverly, MA) to remove cell debris and molecules with molecular weights larger than 3000 and the filtrates were then subjected to HPLC. Cell lysates were prepared and stored at 4°C until shortly before use. The HPLC eluates were collected in 2 ml fractions and each fraction was assayed for content of ITCs and dithiocarbamates by the cyclocondensation assay (27).

Determination of cellular GSH

Reduced GSH levels in cells without exposure to ITCs or their GSH conjugates were measured by either a fluorometric assay (32) as described previously (14) or the Ellman assay (33). In our experiments with Hepa 1c1c7 cells, both assays gave similar values. In cells that were exposed to ITCs or their GSH conjugates, only the Ellman assay was used because the fluorometric assay also measures GSH that is already conjugated with ITCs. In the Ellman assay, cell lysates were treated with 5% trichloroacetic acid to precipitate proteins and the supernatant fraction was diluted with 100 mM potassium phosphate, pH 7.5. The assay was carried out in 1 ml volumes that contained 100–200 µl of the test sample and 700–800 µl of the above buffer. An aliquot of 100 µl of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) reagent in methanol was added to start the reaction and the absorbance was read at 412 nm within 30 s at

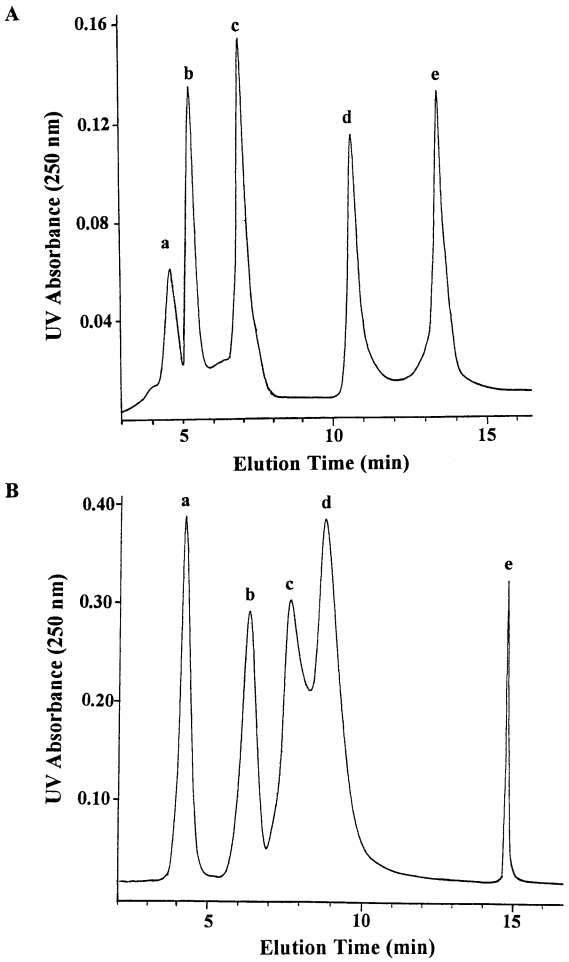

Fig. 2. Paired-ion HPLC separation of sulforaphane and benzyl isothiocyanate and their thiol conjugates. Elution conditions are described in Materials and methods. Retention times: (**A**) cysteinylglycine–SF (a, 4.6 min), GSH–SF (b, 5.4 min), cysteine–SF (c, 7.1 min), *N*-acetylcysteine–SF (d, 10.7 min) and SF (e, 13.0 min); (**B**) GSH–benzyl-ITC (a, 4.3 min), cysteinylglycine–benzyl-ITC (b, 6.4 min), cysteine–benzyl-ITC (c, 7.7 min), *N*-acetylcysteine–benzyl-ITC (d, 8.8 min) and benzyl-ITC (e, 14.9 min).

room temperature with a Cary 1E spectrophotometer. The GSH content was derived from a linear calibration curve established with pure GSH.

Statistics

Except for the statistical analysis shown in Table I, all other reported values are means of two measurements made in two separate experiments, each of which was assayed in duplicate. Replicate analysis routinely had standard errors of $< \pm 7\%$ of the mean.

Results and discussion

SF and benzyl-ITC are accumulated principally as GSH conjugates in cells

As previously observed (14), both SF and benzyl-ITC accumulated rapidly in a dose-dependent manner and to very high concentrations in Hepa 1c1c7 cells (Figure 3). Thus, when these cells were exposed for 30 min at 37°C to a range of concentrations (50–500 μM) of SF, the intracellular levels of 'conjugates' rose to very high levels (5.1–9.4 mM) and approached saturation. Consequently, the internal to external concentration ratio over this range of external concentrations fell from 102- to 19-fold. Under similar conditions, the internal to external concentration ratio for benzyl-ITC over the same range of external concentrations fell from 118- to 31-fold.

Examination of the chemical nature of the intracellularly accumulated products by paired-ion chromatography revealed that at 50–100 μM extracellular SF concentrations, 95–98% of the intracellular species was the GSH conjugate and the remainder (2–5%) was free SF. Three conclusions from these experiments require emphasis: (i) nearly all of the cyclocondensation-positive material in the cell homogenates was recovered in the HPLC fractions; (ii) the vast majority of the accumulated material was the GSH–SF conjugate; (iii) no other dithiocarbamate intermediates of the mercapturic acid pathway were detected (results not shown). When the extracellular SF concentration was raised to 500 μM, the GSH conjugate contributed only 82% of the total conjugates, free SF accounted for 11%, and 7% of the conjugates applied to the column were not eluted in the HPLC fractions. The latter

Table I. GSH levels in Hepa 1c1c7 cells exposed to ITCs or their GSH conjugates

	ITC or its GSH conjugate in medium (mM)	Cellular GSH (mM) (mean ± SD)
Control	0	7.3 ± 0.21
SF	0.05	3.3 ± 0.33
	0.10	2.8 ± 0.36
	0.50	0.8 ± 0.15
Benzyl-ITC	0.05	3.7 ± 0.76
	0.10	2.2 ± 0.17
	0.50	0.9 ± 0.24
GSH–SF	0.06	4.3 ± 0.70
	0.12	3.4 ± 0.57
	0.60	1.5 ± 0.11
GSH–benzyl-ITC	0.05	4.2 ± 0.59
	0.10	3.1 ± 0.12
	0.50	1.7 ± 0.72

Hepa 1c1c7 cells in suspension were incubated with and without the test compound at the indicated concentrations for 30 min at 37°C. The cells were then separated from the medium and lysed by sonication. The cellular content of GSH was measured by the Ellman assay (33) and calculated as intracellular concentration based on cell volume measurements using a calibration line that relates the protein quantity in the lysates to cell volume (14). All measurements were made in quadruplicate.

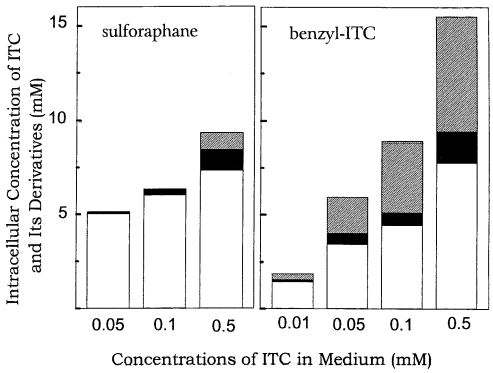

Fig. 3. Chemical nature of SF and benzyl-ITC accumulated in Hepa 1c1c7 cells. Cells in suspension were exposed to SF (left) or benzyl-ITC (right) at the indicated concentrations in medium at 37°C for 30 min and then separated from the medium and sonicated. A portion of each lysate was directly analyzed by paired-ion HPLC (as shown in Figure 2). The original lysate and the HPLC fractions were quantitated by the cyclocondensation assay to determine the total quantities and the amount of each compound in the lysates. The intracellular concentration of each compound was then calculated based on measurement of cell volume. □, GSH–SF or GSH–benzyl-ITC; ■, SF or benzyl-ITC; ▨, SF or benzyl-ITC derivatives whose identities are unknown.

were probably bound to macromolecules which were retained by the column (see below).

Benzyl-ITC was also accumulated in cells largely as the GSH conjugate, although the percentage was lower than that with SF. When Hepa 1c1c7 cells were exposed to the relatively low concentration of 10 μM benzyl-ITC for 30 min at 37°C, the intracellular levels of GSH conjugate attained high levels (1.9 mM), of which 77% was identified as the GSH–benzyl-ITC conjugate. As the intracellular concentrations of the conjugates reached 5.9, 8.9 and 15.5 mM when cells were incubated with 50, 100 and 500 μM benzyl-ITC, respectively, for 30 min at 37°C, the percentage of GSH conjugate in the accumulated material fell correspondingly, but reached a plateau at ~50% (Figure 3). Free benzyl-ITC comprised 5–11% and the remaining portion was not recovered in the HPLC fractions. Thus, although a lower fraction of benzyl-ITC than of SF accumulated as the GSH derivative, this conjugate was nevertheless the major intracellular chemical species, and even when benzyl-ITC accumulated in cells to rather high concentrations, the relative amount of free ITCs remained small. Other intermediates of the mercapturic acid pathway were not detected.

In lysates of cells that were exposed to high concentrations of either SF or benzyl-ITC, a significant portion of the cyclocondensation-positive conjugates detected in the cell lysates and applied to the HPLC columns was not detected by the cyclocondensation assay in the eluted fractions. In order to identify the nature of these unrecovered conjugates (presumably retained on the HPLC column), lysates of cells that were exposed in suspension to 500 μM SF or benzyl-ITC for 30 min were centrifuged through a Centricon filter (hydrophilic membrane, 3000 mol. wt pore size). Of the total conjugates in the cell lysates, only 84% in SF-treated cells and 48% in benzyl-ITC-treated cells were found in the flow-through fraction. Thus, the percentages of conjugates that were retained by the filter were comparable with those that were not recovered in the HPLC fractions (compare with Figure 3). Moreover, when the filtrates were then analyzed by paired-ion HPLC, there was 100% recovery and the chemical compositions were identical to those observed on analysis of the unfiltered lysates. This observation suggests that at high intracellular concentrations, the ITCs accumulated in cells are probably partly associated with cellular macromolecules.

Formation of GSH conjugates at very high concentrations in cells in just 30 min also indicated that the conjugation reaction of each ITC with GSH is likely to be extremely rapid. Although these conjugation reactions can occur non-enzymatically, they may also be catalyzed by glutathione transferases (26,34,35), and the possible involvement of these transferases is being investigated. Our previous studies have shown that the rank order of the initial cellular uptake rates of 1 μM ITCs in Hepa 1c1c7 cells at 4°C [46 (benzyl-ITC) > 22 (allyl-ITC) > 2.7 μM/min (SF)] (34) correlated well with the non-enzymatic second order rate constants of GSH conjugation reactions with the ITCs at 25–30°C and pH 6.5 [130 (benzyl-ITC) > 75 (allyl-ITC) > 45/M/min (SF)] and that depletion of cellular GSH led to significant decreases in ITC accumulation (14). It is therefore very likely that the conjugation of ITCs with GSH is the principal driving force for ITC accumulation, rather than a consequence thereof.

Cellular accumulation of SF and benzyl-ITC depletes GSH

Rapid accumulation of high concentrations of GSH conjugates in cells exposed to either SF or benzyl-ITC was accompanied by depletion of the normally high GSH levels (7.3 mM), as shown in Table I. When Hepa 1c1c7 cells in suspension were exposed to either SF or benzyl-ITC at 50–500 μM for 30 min, GSH levels fell in a dose-dependent manner. In cells that were exposed to the highest concentration of each compound, nearly 90% of cellular free GSH was depleted. Moreover, the extent of depletion of GSH was very similar in SF- and benzyl-ITC-treated cells. Comparison of these results with those shown in Figure 3 demonstrates that the amount of GSH depleted (Table I) was slightly less than the amount of GSH conjugates formed in the cells under similar conditions, suggesting that GSH in cells may be rapidly replenished by synthesis as it is being depleted.

Although exposure of cells to high concentrations of ITCs resulted in rapid depletion of cellular GSH, it is of interest that previous studies have also demonstrated that incubation of cells with low concentrations of ITCs for longer periods of time (e.g. >24 h) leads to a significant elevation of cellular GSH (36). Thus, it seems likely that a cycling mechanism may come into play when cells are chronically exposed to ITCs, i.e. cellular accumulation of ITCs depletes GSH in cells but consequently leads to more biosynthesis of GSH, which in turn could result in more cellular accumulation of ITCs. The molecular mechanism responsible for elevation of cellular GSH by ITCs and other inducers undoubtedly involves up-regulation of γ-glutamylcysteine synthetase, the rate limiting enzyme in the synthesis of GSH. Time- and dose-dependent elevation of the mRNA levels of the catalytic and/or regulatory subunits of γ-glutamylcysteine synthetase was observed when cells were exposed to compounds that raise cellular GSH levels (37,38). The genes encoding both the catalytic (heavy) and regulatory (light) subunits of this enzyme contain anti-oxidant-responsive elements (ARE) in their upstream regions (four AREs in the heavy subunit and one ARE in the light subunit) (38,39). Much evidence has indicated that AREs play a very important role in mediating transcriptional activation of many phase II enzyme genes by various chemical inducers (40,41).

Cellular uptake of GSH conjugates of SF and benzyl-ITC depends on their hydrolysis to free ITCs

Since ITCs appeared to be mainly accumulated as conjugation products with GSH, it became important to examine whether ITCs that were already conjugated with GSH as dithiocarbamates could accumulate in cells and, if so, whether the GSH–ITC conjugates are taken up intact or must first undergo dissociation to their components in order to enter cells. This issue is important because GSH–ITC conjugates inhibit phase I enzymes that activate carcinogens (23), induce phase II enzymes that detoxify carcinogens (our unpublished observations) and inhibit tumorigenesis in animals (24,25). Conjugates of ITCs with GSH and other thiols have been previously shown to dissociate in aqueous systems (42,43) and the dissociation rates of the conjugates determined their activity as inhibitors of several phase I enzymes that may activate carcinogens, suggesting that ITCs might be the active form (44). Exposure of Hepa 1c1c7 cells to GSH conjugates of either SF or benzyl-ITC resulted in rapid accumulation of the conjugates to total intracellular levels that were only slightly lower than those observed upon exposure of cells to equivalent concentrations of free ITCs. As shown in Figure 4, exposure of Hepa 1c1c7 cells in suspension to GSH–SF at 0.05, 0.1 and 0.5 mM for 30 min at 37°C resulted in 64-, 53- and 15-

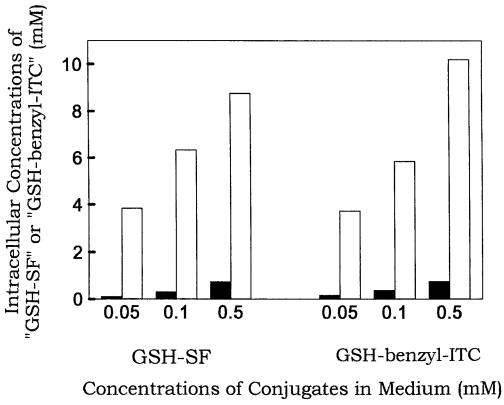

Fig. 4. Inhibitory effect of GSH on the uptake of GSH–SF and GSH–benzyl-ITC conjugates in Hepa 1c1c7 cells. Cells in suspension were incubated for 30 min at 37°C with each conjugate at the indicated concentrations in the medium in the presence (■) or absence (□) of 5 mM GSH. The cells were then separated from the medium and the intracellular concentrations of GSH–SF and GSH–benyl-ITC were determined by the cyclocondensation assay and cell volume measurement.

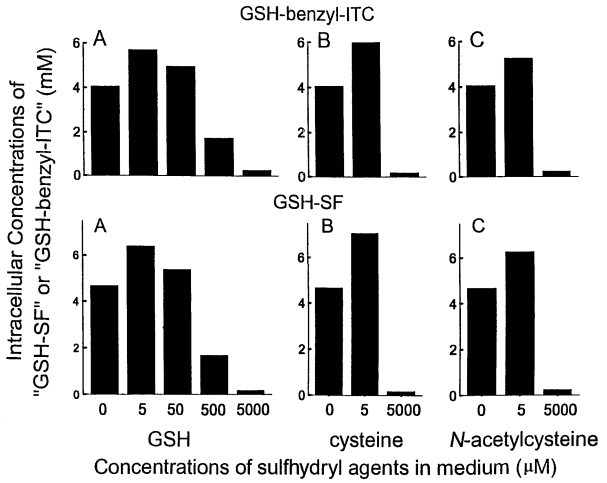

Fig. 5. Effect of a range of concentrations of GSH, cysteine and *N*-acetylcysteine on the uptake of GSH–benzyl-ITC and GSH–SF conjugates in Hepa 1c1c7 cells. Cells in suspension were incubated with GSH–benzyl-ITC (top) or GSH–SF (bottom) at 50 μM in the presence of the indicated concentrations of GSH (**A**), cysteine (**B**) and *N*-acetylcysteine (**C**) in the medium for 30 min at 37°C. Cells were then separated from the medium and the intracellular concentrations of GSH–SF and GSH–benzyl-ITC were determined by the cyclocondensation assay and cell volume measurement.

fold accumulation (3.5–8.8 mM intracellular concentrations), respectively. Interestingly, the extent of GSH–benzyl-ITC conjugate accumulation was similar to that of GSH–SF (Figure 4), unlike the free ITCs, for which cellular accumulation of benzyl-ITC was much higher than that of SF (Figure 3; ref. 14).

To determine whether cellular accumulation of GSH–SF and GSH–benzyl-ITC depended upon their dissociation, each conjugate was incubated with the medium and the mixtures were analyzed by paired-ion HPLC and the cyclocondensation assay. Both GSH–SF and GSH–benzyl-ITC dissociated significantly to free ITCs in the medium. For example, when 0.1 mM concentrations of GSH–SF and GSH–benzyl-ITC were incubated in medium for 30 min at 37°C, 46 and 22%, respectively, of the added compounds were converted to free ITCs. Since dissociation of GSH–benzyl-ITC was significantly slower than that of GSH–SF, the similar cellular accumulation levels of GSH–benzyl-ITC and GSH–SF observed above may result from a complex interplay of the kinetics of dissociation, uptake and reconjugation.

The net free ITC concentration resulting from dissociation of the GSH conjugates was significantly reduced by excess GSH in the medium. For example, when 0.1 mM concentrations of each conjugate were incubated in medium for 30 min at 37°C in the presence of 5 mM GSH, only 2–3% of the conjugates were converted to free ITCs. Interestingly, under the same experimental conditions (0.1 mM conjugate, 5 mM GSH, 30 min at 37°C), cellular accumulation of the conjugates was also drastically reduced; intracellular concentrations reached only 0.10–0.75 mM, i.e. 1.2–2.5 times those of the initial extracellular concentrations (Figure 4).

The following experiments were designed to understand how changes in extracellular concentrations of GSH affect accumulation of the conjugates and whether other sulfhydryl agents might also affect this accumulation. Varying the GSH concentration in the medium resulted in a biphasic effect on GSH–SF and GSH–benzyl-ITC accumulation. When GSH was raised from 50 μM to 5 mM at a constant concentration of 50 μM of the conjugates, intracellular accumulation of both conjugates was dramatically depressed in a concentration-dependent manner (Figure 5A). Interestingly, both cysteine and *N*-acetylcysteine showed inhibitory effects very similar to those of GSH on cellular accumulation of the conjugates (Figure 5B and C). However, at low GSH concentrations (5 and 50 μM), the intracellular accumulation of conjugates was 37–41 and 15–22%, respectively, higher than that in the absence of added GSH (Figure 5A). Likewise, low concentrations of either cysteine or *N*-acetylcysteine in the medium (5 μM) enhanced cellular accumulation of each conjugate by 49–51% (cysteine) and 30–34% (*N*-acetylcysteine) (Figure 5B and C). In the above experiments, only the highest and lowest concentrations of cysteine and *N*-acetylcysteine (5 mM and 5 μM) were tested since this is sufficient to compare the effects of these compounds with that of GSH. The precise mechanism of this phenomenon is unclear. Since free ITCs hydrolyzed from their GSH conjugates in the medium are most likely reconjugated with cysteine or *N*-acetylcysteine when these compounds are present in excess in the medium, the result suggested that in addition to GSH–ITC conjugates, cysteine and *N*-acetylcysteine conjugates are also unlikely to be significantly accumulated intact by cells. The effect of each thiol appears to be to displace the equilibrium of hydrolysis of each GSH–ITC to the side of the conjugates.

Further studies have shown that the effects of GSH, cysteine and *N*-acetylcysteine on cellular accumulation of the conjugates shown above are almost identical to the effects of these agents on cellular uptake of free ITCs. In the presence of 5 mM concentrations of each agent in the medium, cellular accumulation of SF and benzyl-ITC was almost completely blocked, whereas low concentrations of each agent (5 μM) significantly enhanced the accumulation of each ITC by 20–27% (Figure 6). Moreover, a dose-dependent biphasic effect of GSH on cellular uptake of ITCs was also observed (Figure 6A). Thus, the profound inhibition of cellular uptake of the conjugates and ITCs by GSH, cysteine and *N*-acetylcysteine strongly suggests that only very limited (if any) direct cellular uptake of the conjugates takes place and that the conjugates readily dissociate to free ITCs that are then taken up by cells. The limited accumulation of the conjugates in the presence of excess thiols is probably attributable to incomplete inhibition of dissociation of the conjugates. The proposed conclusion

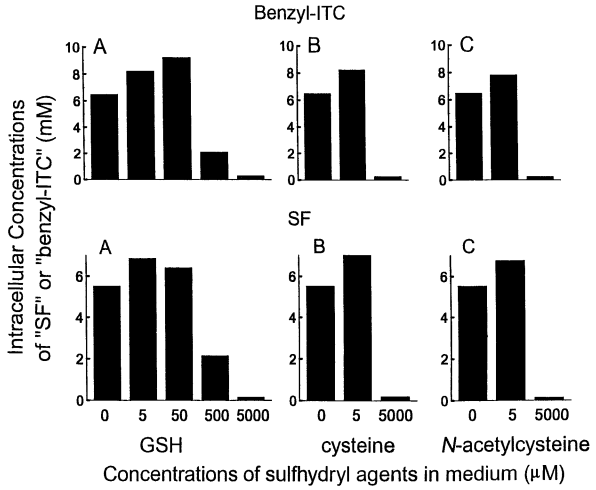

Fig. 6. Effect of a range of concentrations of GSH, cysteine or *N*-acetylcysteine on the uptake of benzyl-ITC and SF in Hepa 1c1c7 cells. Cells in suspension were incubated with 50 μM benzyl-ITC (top) or SF (bottom) in the presence of the indicated concentrations of GSH (**A**), cysteine (**B**) and *N*-acetylcysteine (**C**) in the medium for 30 min at 37°C. Cells were then separated from the medium and the intracellular concentrations of SF and benzyl-ITC were determined by the cyclocondensation assay and cell volume measurement.

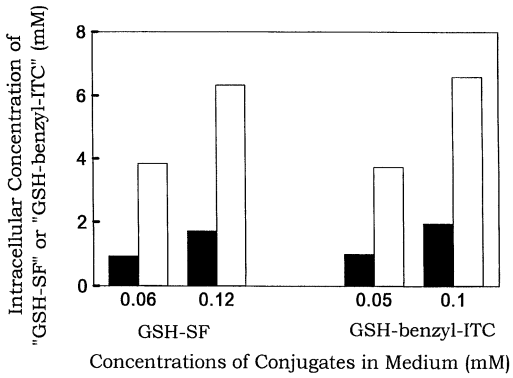

Fig. 7. Effect of lowering cellular GSH on the uptake of GSH–SF and GSH–benzyl-ITC conjugates in Hepa 1c1c7 cells. Cells in monolayers were treated (■) or untreated (□) with 100 μM BSO for 24 h and then trypsinized and suspended in medium. The suspension was incubated with each conjugate at the specified concentration with (for BSO-treated cells) or without (for non-BSO-treated cells) 100 μM BSO for 30 min at 37°C. Cells were separated from the medium and the intracellular concentrations of GSH–SF and GSH–benzyl-ITC were determined by the cyclocondensation assay and cell volume measurement. Treatment of cells with 100 μM BSO for 24 h depleted cellular GSH by 80%.

that free ITCs but not their GSH conjugates are taken up by cells is in agreement with the findings shown below that uptake of the conjugates depends on cellular GSH levels and subsequently leads to depletion of GSH. The above observations are also consistent with previous findings that addition of excess GSH or other thiols to medium containing ITCs significantly reduced cytotoxicity of the latter compounds (42).

Cellular uptake of GSH conjugates of SF and benzyl-ITC depends on the GSH content of cells and leads to depletion of GSH

We previously reported that cellular accumulation of free ITCs was inhibited in cells depleted of GSH by treatment with BSO (14). Treatment of Hepa 1c1c7 cells for 24 h with 100 μM BSO, which inhibits γ-glutamylcysteine synthetase (45), depleted cellular GSH by 80%. Exposure of such BSO-treated cells in suspension to each GSH–ITC conjugate for 30 min at 37°C resulted in a decrease in cellular GSH–ITC accumulation by 70–76%, in comparison with that of cells not treated with BSO (Figure 7). Moreover, exposure of cells to GSH–ITC conjugates also significantly reduced cellular GSH levels in a dose-dependent manner (Table I). At the highest conjugate concentrations, cellular GSH was reduced to ~20% of the level in control cells, which was only slightly higher than that observed in cells exposed to free ITCs (Table I). Since it seems unlikely that uptake of intact GSH–ITC conjugates would deplete GSH or depend on the GSH concentration in the cells, these experiments provided additional evidence that ITCs rather than GSH–ITCs are taken up by cells. Thus, it can be concluded that the concentrative accumulation of GSH–ITC conjugates in Hepa 1c1c7 cells results mostly, if not completely, from hydrolysis of the conjugates to free ITCs, which are the chemical species taken up by cells.

GSH conjugates of SF and benzyl-ITC formed in cells are rapidly metabolized

In order to understand how the intracellularly accumulated GSH–ITC conjugates are further metabolized or disposed of, Hepa 1c1c7 cells in monolayers were exposed to 10 μM benzyl-ITC or 20 μM SF for 6 h at 37°C; the intracellular concentrations of 'benzyl-ITC' and 'SF' at this time were 0.35 and 1.67 mM, respectively. This result is consistent with our earlier observation that accumulated intracellular 'benzyl-ITC' disappeared more rapidly from cells than 'SF' (14). Examination of higher intracellular ITC concentrations or longer incubation times was difficult because of cytotoxicity. When the cell lysates were analyzed by paired-ion HPLC, only 4–8% of the total 'SF' and 'benzyl-ITC' in cells were GSH conjugates, indicating that the GSH–ITC conjugates that initially formed in Hepa 1c1c7 cells were further metabolized or released from the cells. Nevertheless, free ITCs and other known intermediates of the mercapturic acid pathway were not detected.

In an attempt to determine the metabolic fate of the GSH conjugates, the lysates were centrifuged through a Centricon filter (hydrophilic membrane, 3000 mol. wt exclusion size). Whereas 57% of the total 'SF' in the lysates was found in the filtrate, only 13% of the 'benzyl-ITC' in the lysates passed through the filter. Since the ITC derivatives retained by the filter are likely to have molecular weights >3000, this result suggests that a significant portion of the GSH–ITC conjugates that formed initially became bound to cellular macromolecules. Although the identities of these macromolecules are not known, conjugation reactions of GSH with ITCs are known to be reversible and both the conjugates and free ITCs may react with a variety of cellular nucleophiles, especially with protein sulfhydryl groups.

Although the amount of 'benzyl-ITC' that passed through the filter (13%) matched the amount of the GSH conjugate in the lysates (8%), the amount of 'SF' in the filtrate (57%) far exceeded that of the GSH conjugate in the lysates (4%), suggesting that a significant portion of cellular GSH–SF was metabolized to forms that have molecular weights <3000. The exact chemical nature of these metabolites in the cell is unknown, but it is possible that they include the *S*-oxide reduction product of SF (erucin) and a product of dehydrogenation of the methylene bridge, as reported in the rat (46).

Because both GSH–SF and GSH–benzyl-ITC are rapidly

converted to unknown species in Hepa 1c1c7 cells, the ultimate chemical forms of SF and benzyl-ITC responsible for inducing phase II enzymes remain unclear. However, it has been recognized that nearly all of the phase II enzyme inducers (including ITCs) are electrophilic or can be converted to electrophiles in cells and that their inducer activity is related to their chemical reactivity with sulfhydryl groups (47,48). Since ITC conjugation reactions with thiols such as GSH are reversible (43) and the conjugates also readily undergo exchange reactions with free thiols (42), it is possible that both free ITCs and their thiol conjugates (including GSH conjugates) are ultimate phase II enzyme inducers. The cellular 'sensor' of phase II enzyme inducers has not been clearly identified, although a DNA-responsive element, namely the ARE, has been shown to mediate, at least partly, the transcriptional activation of phase II enzyme genes (41,49). Recently, Itoh *et al*. have suggested that transcription factor Nrf2 and its suppressor Keap1 might be the cellular 'sensor' (50). They showed that Nrf2 is associated with Keap1 in the cytoplasm but that electrophilic inducers appear to antagonize Keap1, allowing Nrf2 to dissociate from it. The free Nrf2 then dimerizes with the small Maf protein to activate the ARE (50). The Nrf2–small Maf complex was found to bind to the ARE with high affinity and phase II enzyme induction was abolished in Nrf2 knock-out mice (51).

Summary

Exposure of Hepa 1c1c7 murine hepatoma cells to SF or benzyl-ITC as well as their GSH conjugates leads to rapid accumulation to very high concentrations, principally in the form of GSH conjugates. Evidence is presented that the conjugation reactions are likely to be the driving force for cellular accumulation of ITCs. Accumulation of ITCs depends on cellular GSH levels and results in depletion of GSH. Exposure of cells to GSH conjugates of SF and benzyl-ITC also results in accumulation of high intracellular levels of these conjugates. However, it is very likely that these conjugates are not absorbed into cells intact but are dissociated to ITCs which then enter the cells. These findings provide insight into the mechanisms by which ITCs and their thiol conjugates accumulate in cells and will lead to a better understanding of the phase II enzyme inducer potencies and anticarcinogenic properties of this class of compounds.

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