Decomposition Rates of Isothiocyanate Conjugates Determine Their Activity as Inhibitors of Cytochrome P450 Enzymes†

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Thiol conjugates of isothiocyanates (thiol-ITCs) are metabolites of ITCs formed in the mercapturic acid pathway in mammals. They are effective chemopreventive agents in mouse lung tumor bioassays and in other models. Thiol-ITCs are inhibitors of P450s, but it has not been determined if P450 inhibition is due to conjugates themselves or to parent ITCs released by deconjugation reactions. In studies of mechanism of chemopreventive action of thiol-ITCs, rates of deconjugation of Cys, GSH, and *N*-acetyl-L-cysteine (*N*AC) conjugates of benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), 6-phenylhexyl isothiocyanate (PHITC), and sulforaphane (SFN), expressed as the first-order rate constant k_1 and the half-life of decomposition $Dt_{1/2}$, were measured in aqueous solutions at pH 7.4 and 37°. The $Dt_{1/2}$s for the Cys conjugates were severalfold shorter than the $Dt_{1/2}$s for respective GSH conjugates, while the $Dt_{1/2}$s for the *N*AC conjugates were the longest. Cleavage of thiol conjugates was pH dependent, much slower under acidic conditions than at pH 7.4. Inhibition of P450 enzymes by thiol-ITCs was followed using PROD (pentoxyresorufin O-dealkylation) for P450 2B1 and EROD (ethoxyresorufin O-dealkylation) for P450 1A1. The inhibition of PROD and EROD by aqueous thiol-ITCs increased with preincubation time and was roughly parallel to the extent of decomposition of the conjugate that had occurred, indicating that both potency of the respective parent ITC and the rate of reductive cleavage of the conjugate influenced enzyme inhibition. In the presence of 250−1000 μM GSH, comparable to physiological levels, rates of deconjugation of thiol-ITCs were markedly reduced; inhibition of PROD was also proportionately reduced. Slow rates of decomposition of thiol-ITCs anticipated in plasma and tissues suggests that inhibition of P450 enzymes involved in carcinogen activation by ITCs released from thiol-ITCs may not be a principal mechanism for their tumor inhibitory activity; other mechanisms probably contribute to their chemopreventive activity.

Introduction

Isothiocyanates (ITCs)[1] are electrophiles; they react predominantly with thiols, and, to a much lesser extent, with NH_2 and OH groups. ITCs also bind covalently with cellular proteins (*1*). The inhibition of metabolism of procarcinogens to more reactive species by inactivation of P450 enzymes is, at least in part, responsible for the chemopreventive activity of ITCs in some animal models (*2*, *3*).

Thiol conjugates of ITCs are formed by nonenzymatic and enzymatic conjugation with GSH as a major route of metabolism (*4*). Stepwise enzymatic removal of glutamine and glycine from GSH yields L-cysteine-ITC conjugates (ITC-Cys), which are subsequently acetylated to yield *N*-acetyl-L-cysteine (*N*AC) conjugates of ITCs (mercapturic acids) excreted in the urine (*4*−*7*). *N*AC conjugates of ITCs display chemopreventive activity in tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo(*a*)pyrene [B(a)P] induced A/J mouse lung tumor bioassays (*8*−*10*), and they inhibit azoxymethane (AOM)-induced colonic aberrant crypt foci formation in Fischer 344 rats (*11*). Thiol conjugates of ITCs have also been reported to inhibit growth of cultured human cancer cells in culture (*12*−*14*). Like ITCs, upon incubation with microsomes, thiol conjugates of ITCs inhibit the enzymes of P450 that metabolize NNK and other carcinogens, but their activities are considerably weaker than free ITCs (*15*−*18*).

Consistent with their weaker inhibition of P450 in in vitro studies (*18*), the potency of thiol conjugates of ITCs as chemopreventive agents is less than that of their respective parent ITCs (*10*, *16*). However, the greater solubility of thiol-ITCs in aqueous media and their lower toxicity and pungency, compared with the parent ITCs, suggests that thiol conjugates would be a preferred form for clinical chemoprevention trials.

The mode of action of thiol conjugates as chemopreventive agents is not yet known. However, it has been

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[1] Abbreviations: AITC, allyl ITC; AOM, azoxymethane; B(a)P, benzo[*a*]pyrene; BITC, benzyl ITC; $Dt_{1/2}$, half-life of decomposition; IC_{50}, concentration for 50% inhibition of enzyme activity; $It_{1/2}$, half-life of inhibition; ITC, isothiocyanate; ITC-Cys, L-cysteine conjugate of ITC; ITC-GSH, glutathione conjugate of ITC; ITC-*N*AC, *N*-acetyl-L-cysteine conjugate of ITC; *N*AC, *N*-acetyl-L-cysteine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PEITC, phenethyl isothiocyanate, PHITC, 6-phenylhexyl isothiocyanate; SFN, sulforaphane; thiol-ITC, thiol conjugate of isothiocyanate.

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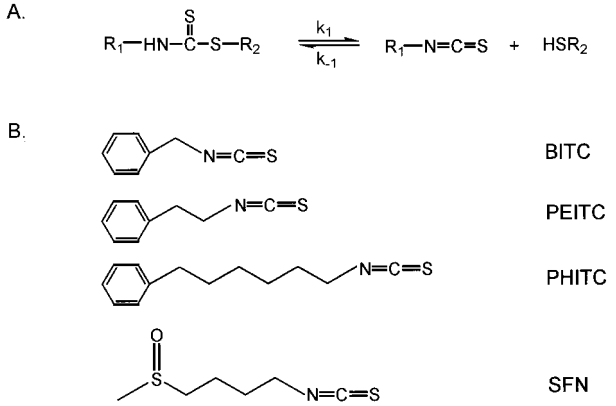

Figure 1. (A) Dissociation of a typical ITC-thiol conjugate in aqueous media. (B) Structures of ITCs selected for study.

shown that thiol conjugates of ITCs in aqueous solution are reductively cleaved to yield the ITC and thiol (Figure 1A). It has been speculated that decomposition of ITC-thiol conjugates to yield free ITCs may be a requisite for the chemopreventive activity of the conjugates. Indirect evidence supporting such a proposition include the observations that thiol conjugates are less potent inhibitors than the parent ITCs (*18*), and that a functional isothiocyanate group is necessary for the chemopreventive activity of ITCs (*19*). In the present work, using thiol conjugates of benzyl ITC (BITC), phenethyl ITC (PEITC), 6-phenylhexyl ITC (PHITC), and sulforaphane (SFN), we provide evidence that ITCs released from conjugates rather than ITC conjugates themselves inhibit P450 enzymes by comparing the rates of decomposition of ITC-thiol conjugates with their potency as inhibitors of P450 1A1 and P450 2B1. BITC, PEITC, and SFN were chosen for study because they are major ITC constituents of garden cress, watercress, and broccoli, respectively, common cruciferous vegetables consumed by humans (*21*). PHITC is a highly lipophilic synthetic homologue that is remarkably potent as a chemopreventive agent in NNK induced A/J mouse lung tumor bioassays (*22*).

Experimental Procedures

Instruments. A Waters HPLC system (Waters Corp., Milford, MA) equipped with an automated gradient controller and two Waters 501 pumps were utilized. Analysis at 254 nm was performed using a Shimadzu UV−vis spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) and a model 2 IN/US β Ram (IN/US Systems, Inc, Tampa, FL) for HPLC data acquisition. For monitoring of thiol conjugate deconjugation, an isocratic mobile phase consisting of 0.1% trifluoroacetic acid (TFA) in water:acetonitrile (60:40) was used (1 mL/min) with two 4.6 × 250 mm columns in tandem (*17*). A Perkin-Elmer Model 540-30 fluorescence spectrometer (Perkin-Elmer Corp., Norwalk, CT) was utilized for the P450 enzyme assays. The assay cuvettte for PROD and EROD was maintained at 37 °C by means of a Haake model B3-C10 circulating waterbath (Fisher Scientific, Pittsburgh, PA).

Chemicals. BITC, PEITC, and *N*AC were purchased from Aldrich Chemical Co. (Milwaukee, WI), while GSH, Cys, pentoxyresorufin, and ethoxyresorufin were purchased from Sigma (St. Louis, MO). PHITC and the GSH and Cys conjugates of BITC, PEITC, and PHITC were synthesized and characterized in our laboratory (*21*); PHITC-Cys, BITC-*N*AC, PEITC-*N*AC, and PHITC-*N*AC were synthesized by the Organic Synthesis Facility of the American Health Foundation, and purity and structure were verified by HPLC and NMR, respectively (*17*). The d,L-sulforaphane [1-isothiocyanato-4-(methylsulfinyl) butane, SFN] was purchased from LKT Laboratories, Inc. (St. Paul, MN); purity (>98%) was determined by HPLC. The *N*AC, Cys, and GSH conjugates of SFN were prepared as described previously (*20*). SFN conjugates were purified by HPLC, and structures were confirmed by direct electrospray MS in the positive ion mode (m/z [MH^+] 341, 298.9, and 485, respectively). Purity was >98% by HPLC for the SFN-*N*AC and SFN-GSH, and >96% for SFN-Cys. Other chemicals, substrates, and cofactors were of highest purity commercially available.

Microsomes. For induction of microsomal enzymes, male F344 rats, 8 weeks of age, were treated i.p. with 75 mg/kg b.w. phenobarbital for 3 days (PROD) or 20 mg/kg 3-methylcholanthrene for 3 days (EROD). The rats were then sacrificed and microsomes were prepared (*18*). Microsomal suspensions (10−12 mg of protein/mL) were assayed using the Coomassie plus protein reagent (Pierce Chemical Co., Rockford, IL) and stored in aliquots at −80 °C until utilized; the microsomes were then diluted to 2 mg of protein/mL with 0.1 M Tris buffer (pH 7.8) and kept on ice prior to use the same day.

First-Order Rate Constant and Half-Life of Decomposition of Thiol Conjugates of ITCs. A solution of each thiol conjugate was prepared as rapidly as possible by dilution of 0.2 mL of 5 mM thiol conjugate freshly prepared in 20 mM sodium phosphate buffer (pH 7.4):DMSO (1:1) with 20 mM phosphate buffer (pH 7.4) to 10 mL, making a 100 μM conjugate solution in 1% DMSO. DMSO was provided to facilitate the solubility of the thiol-ITC conjugates in aqueous medium. An aliquot (50 μL) was immediately analyzed by HPLC (0 min time point), and the peak area was determined. The remainder of the ITC conjugate solution was incubated at 37 °C in a shaking waterbath; at intervals of 20−30 min, a 50 μL aliquot was analyzed by HPLC. A semilog plot of the initial slope for the disappearance of the conjugate was used to calculate k_1, the first-order rate constant for the decomposition reaction. A linear plot of the conjugate peak area remaining at each time point versus minutes was generated to determine the half-life of decomposition ($Dt_{1/2}$), the time required for decomposition of half of the thiol conjugate.

Inhibition of Isozymes of CYP. Assays for PROD and EROD were conducted using phenobarbital-induced or 3-methyl cholanthrene-induced microsomes, respectively (*23*, *24*). Assay tubes containing 0.1 M Tris buffer (pH 7.8) and 10 μL of 500 mM $MgCl_2$ were prewarmed at 37 °C. At the initiation of each assay, a 100 μM solution of ITC-conjugates in 1% DMSO:20 mM phosphate (pH 7.4) was prepared as rapidly as possible by the method identical to that employed in assays for determination of decomposition half-life described above. An aliquot, amount depending on the final assay concentration selected, was immediately added to the assay tube, while the remaining ITC-conjugate solution was incubated at 37 °C. Substrate (10 μL of 2 mM pentoxyresorufin for PROD or 10 μL of 0.34 mM ethoxyresorufin for EROD in DMSO) and microsomes (20 μg in 10 μL Tris buffer, pH 7.8) were rapidly added to the assay tube, mixed, and then carefully poured into a quartz cuvette. The baseline fluorescence (excitation, 530 nm, slit 5 nm and emission, 585 nm, slit 5 nm) was then monitored using a Recordall Series 5000 x−y recorder (Fisher Scientific, Pittsburgh, PA) for approximately 2.5 min prior to addition of NADPH (50 mM in 10 μL water, final concentration 250 μM) to activate the assay reaction (total assay volume 2 mL), which was mixed and followed for 3−4 min (*18*, *23*, *24*). The initial slope of the experimental fluorescence curve was measured by drawing a tangent line with origin at the intercept of an extension of the baseline fluorescence plot with the experimental curve. The slope of baseline fluorescence was subtracted from the initial slope of the experimental fluorescence curve to determine enzyme activity (pmol/min/μg of microsomal protein). For each set of assays, the gain on the fluorescence spectrometer was standardized as closely as possible to 2 pmol/chart unit using a 1 mM resorufin standard in DMSO diluted 20 000 times with 0.1 M Tris buffer (pH 7.8) just prior to standardization (resorufin solutions are light sensitive, and were stored in the dark at −20

Table 1: Hydrolysis of Isothiocyanate Conjugates and Inhibition of PROD; 100 μM Solutions in 20 mM PO_4 Buffer/DMSO AT 37 °C

compd	$k_1 \times 10^2$ (min^{-1})[a]	half-life (min) $Dt_{1/2}$ (decomposition)[b]	half-life (min) $It_{1/2}$ (inhibition)[c]
BITC-Cys	6.7	12	20.5
BITC-GSH	0.86	67	130 (est.)
BITC-*N*AC	0.36	157	160 (est.)
PEITC−Cys	4.1	12.5	10
PEITC-GSH	0.99	58	47
PEITC-*N*AC	0.49	100	90
PHITC−Cys	0.91[d]	45.3 (14)[d]	18
PHITC-GSH	0.81	57 (37)	11
PHITC-*N*AC	0.33	219 (276)	140 (est.)
SFN−Cys	2.9	14	ND[e]
SFN-GSH	0.81	58	ND
SFN-*N*AC	0.37	173	ND
PEITC-GSH + 250 μM GSH	0.13	>300	ND
PEITC-*N*AC + 250 μM GSH	0.14	>300	ND

[a] k_1, first-order rate constant for decomposition. [b] $Dt_{1/2}$, half-life of decomposition in DMSO:20 mM PO_4 buffer (pH 7.4) (1:99 vol/vol). [c] $It_{1/2}$, half-life of inhibition of PROD at stipulated concentrations: 10 μM for BITC conjugates, 4 μM for PEITC conjugates, and 0.2 μM for PHITC conjugates. [d] K_1 and $Dt_{1/2}$ determined in DMSO: 20 mM PO_4 buffer (pH 7.4) (15:85 vol/vol). [e] ND: not determined. Values shown are representative of two to five experiments performed for each conjugate.

°C). For the PROD assay, the volume of the 100 μM ITC-thiol conjugate aliquot added to each assay cuvette was selected so that the final assay concentration would be approximately twice the previously published IC_{50} of the respective parent ITC (*18*). Thus, assay concentrations of 10, 4, and 0.2 μM for the thiol conjugates of BITC, PEITC, and PHITC, respectively, were determined to require 200, 80, and 4 μL, respectively, per assay. An appropriate aliquot of phosphate buffer containing 1% DMSO was used for the 0 min (baseline) assay. After consideration of volumes required [200 μL, 1 M Tris (pH 7.8) and 10 μL each of Mg^{2+}, substrate, microsomes, and NADPH] and the EROD IC_{50} for PEITC (*18*), 88 μM (1760 μL of 100 μM conjugate) was selected as the appropriate final concentration of thiol conjugates for the EROD assay. The corrected rate of resorufin formation in the assays at each time point (3, 10, 20, 40, 60, 90, and 120 min) was compared to the 0 min activity to calculate the degree of inhibition of P450 enzyme activity.

Results

First-Order Rate Constant and Half-Life of Decomposition of Thiol Conjugates of ITCs. The first-order rate constant k_1 and the $Dt_{1/2}$ for each of the GSH, Cys, and NAC conjugates of BITC, PEITC, PHITC, and SFN at pH 7.4, 37 °C, are presented in Table 1. The value of k_1 was roughly inversely proportional to $Dt_{1/2}$. Preparations of the PHITC conjugate solutions, especially PHITC-Cys, were turbid in 1% DMSO:buffer, indicating only partial solubility, hence those assays were also conducted in 15% DMSO:20 mM phosphate buffer (pH 7.4). Figure 2 shows a typical series of chromatograms for the decomposition of PEITC-GSH at pH 7.4, which demonstrates the time-dependent formation of free PEITC and GSH as products of cleavage of the conjugate. Decomposition of the Cys conjugates of all of the ITCs studied was quite facile, several times more rapid than that of the GSH-ITCs. In the same ITC series, the $Dt_{1/2}$s for the NAC-conjugates were consistently longer than that of the respective Cys- and GSH-conjugates, indicating their greater stability in aqueous media. Interestingly, whereas the respective $Dt_{1/2}$s of GSH and Cys conjugates of all ITCs studied showed close agreement, the stability of PHITC-NAC was significantly greater than that of NAC conjugates of the arylalkylisothiocyanates with shorter alkyl chain lengths (C_1 or C_2). To demonstrate whether the cleavage of ITC-thiol conjugates is influenced by GSH at physiological concentrations, decomposition of PEITC-GSH was compared with that of PEITC-GSH in the presence of 250 μM GSH (Table 1 and Figure 3); the $Dt_{1/2}$ was increased by more than 5-fold, while k_1 was reduced by approximately the same factor. De-conjugation of PEITC-NAC was also inhibited severalfold by addition of 250 μM GSH to the incubation medium ($Dt_{1/2}$ > 300 min compared with $Dt_{1/2}$ of 100 min for PEITC-NAC in the absence of thiol). Higher concentrations of GSH further decreased the rates of decomposition of thiol conjugates (data not shown).

Effect of pH on Decomposition Half-Life. The pH of the incubation medium was changed to simulate physiological conditions in the stomach (gastric juice, pH ~2.0), kidney (tubular filtrate, pH ~5.0), and to investigate cleavage of conjugates under basic conditions (pH 10.0). Acidic media substantially retarded the rates of decomposition of SFN-*N*AC and PEITC-*N*AC, while basic conditions greatly enhanced decomposition (Figure 4). Decomposition of the conjugates were also temperature dependent, and occurred at slower rates at lower temperatures (data not shown).

Time Course of Inhibition of PROD and EROD by ITC Conjugates. Microsomal enzyme activity of PROD monitored by fluorescence assays was rapidly inhibited in a time-dependent manner after addition of final concentrations of 10, 4, and 0.2 μM BITC-, PEITC-, and PHITC-conjugates, respectively. The potency of arylalkyl ITCs as inhibitors of PROD has been demon-

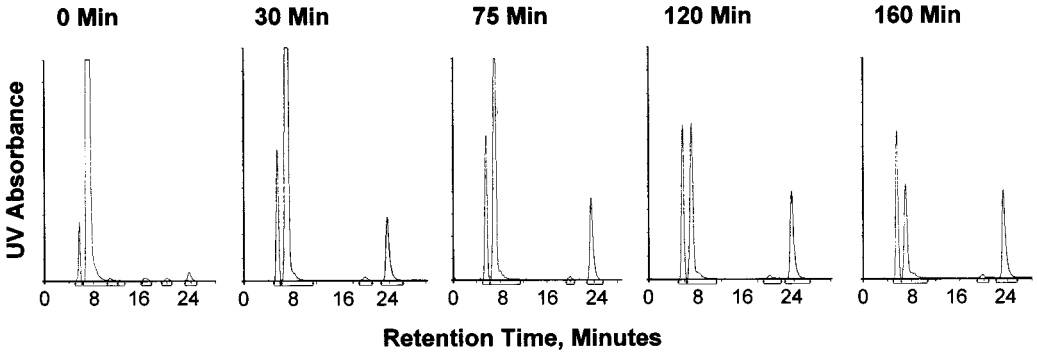

Figure 2. Time course of decomposition of PEITC-GSH; chromatograms of 50 μL aliquots at specific time points. Retention time for PEITC-GSH is 7.3 min; peaks at 5.9 and 23.9 min are GSH and PEITC, respectively.

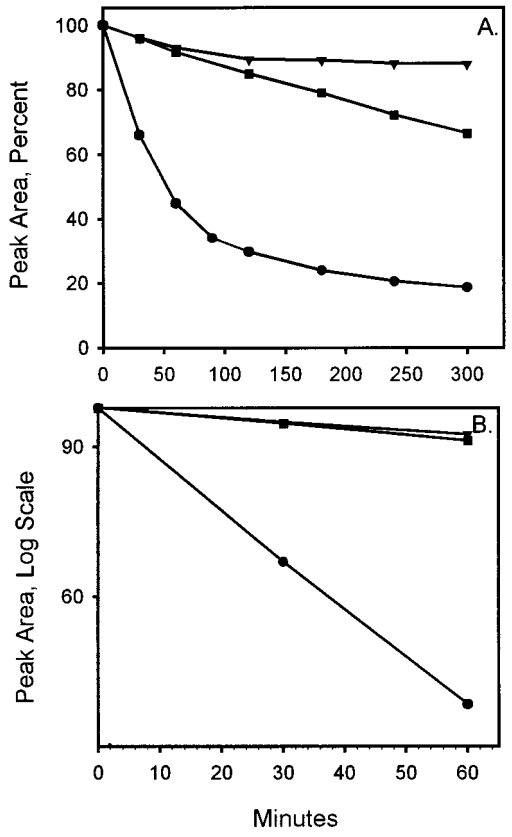

Figure 3. Effects of 250 μM GSH on the decomposition of PEITC-GSH and PEITC-*N*AC. (A) Linear plot for determination of $Dt_{1/2}$. (B) Semilog plot for calculation of k_1. (●) 100 μM PEITC-GSH (no GSH); (▼) 100 μM PEITC-GSH plus 250 μM GSH, (■) 100 μM PEITC-NAC plus 250 μM GSH.

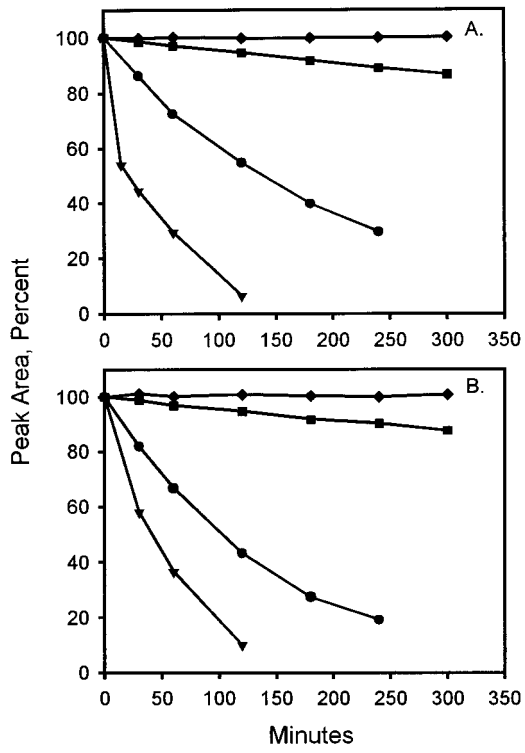

Figure 4. Effect of pH on the decomposition of *N*AC conjugates of ITCs at 37°. (A) PEITC-*N*AC (B) SFN-*N*AC: (◆) pH 2.0, (■) pH 5.0, (●) pH 7.4, (▼) pH 10.0.

strated to greatly increase as the alkyl chain length increased (PHITC ≫ PEITC > BITC) (*18*). Raw data for

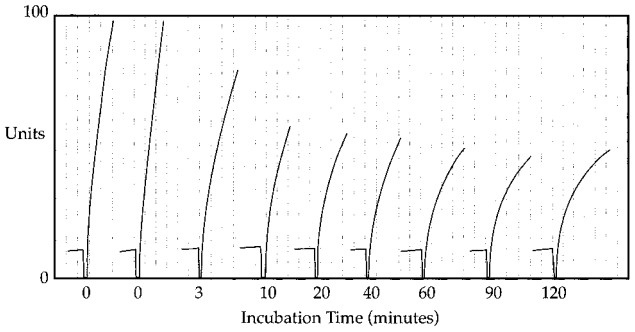

Figure 5. Raw data for assay of effect of PEITC-GSH on PROD activity; At 0 min, PEITC-GSH was dissolved in 20 mM PO_4 buffer, pH 7.4 containing 1% DMSO; at each time point 80 μL aliquots of PEITC-GSH (final concentration 4 μM) were assayed.

a typical experiment, the inhibition of PROD by PEITC-GSH at 4 μM, are shown in Figure 5. As the conjugate in solution decomposed, products were formed that inhibited PROD activity. The increasingly curvilinear assay traces are typical of substrate inhibition in this assay (*25*). In all instances, the *N*AC conjugates were less potent inhibitors of PROD than the respective GSH conjugates (Figure 6). The Cys conjugates were the most potent inhibitors, with the only exception being PHITC-Cys. PHITC conjugates were analyzed at 0.2 μM; at such a low concentration, other factors, such as incomplete solubility and possible deposition on the glass surface of the incubation vial and cuvette, may have compromised the assay results. Nevertheless, a time dependent increase in inhibition of PROD was seen for each experiment. Inhibitory potency was expressed as the $It_{1/2}$, the time required for 50% inhibition of control PROD activity (Table 1). When thiol conjugates of PEITC were added to the EROD assay at specific time points during their course of decomposition (Figure 7), the order of $It_{1/2}$ (Cys > GSH > *N*AC) was identical to the observed for the $Dt_{1/2}$. Control assays for *N*AC, GSH, or Cys, when incubated at 100 μM concentrations and assayed at each time point at a final concentration of 88 μM in a manner identical to that for ITC-thiols, failed to demonstrate appreciable inhibition at the time points assayed. The high concentrations (88 μM) of PEITC conjugates used in the assay for EROD reflect the relatively low inhibitory potency of PEITC (IC_{50} = 47 μM) for P450 1A1/2 (*18*). When GSH at concentrations of 100, 250, or 1000 μM was added to 100 μM solutions of PEITC-GSH or PEITC-*N*AC in 20 mM phosphate buffer (pH 7.4) containing 1% DMSO, the activity of the conjugates as inhibitors of PROD decreased in a dose-dependent manner (Figure 8). The initial drop in PROD enzyme activity assays was attributed to the small amount of DMSO present in the assays (final concentration, 0.04%), which inhibits PROD activity (data not shown). DMSO was not present in the control assays (0 min).

Discussion

Our results demonstrate that thiol conjugates of ITCs in aqueous solution exist in equilibrium with the free form; thiol conjugates are deconjugated over time to yield ITCs and thiols until a state of equlibrium is reached. The rates of cleavage appeared to depend on the structures of both the respective thiol moieties and the ITCs. We have also demonstrated that as the conjugates decompose, their respective potencies as inhibitors of 450

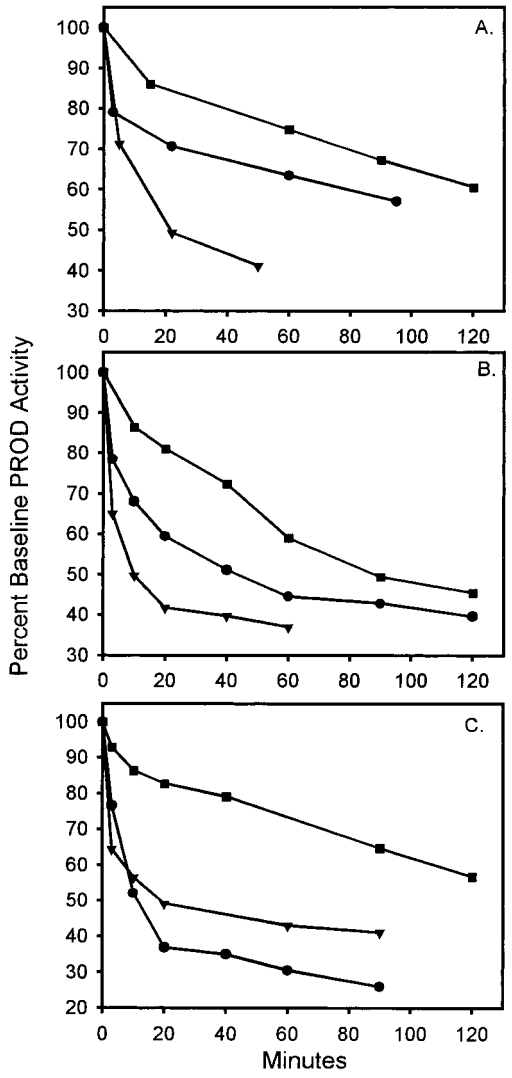

Figure 6. Time course of inhibition of PROD by thiol conjugates of ITCs at 37°. (A) BITC conjugates, final assay concentration, 10 μM; (B) PEITC conjugates, final assay concentration 4 μM; C. PHITC conjugates, final concentration 0.2 μM. (■) *N*AC conjugates, (●) GSH conjugates, (▼) Cys conjugates.

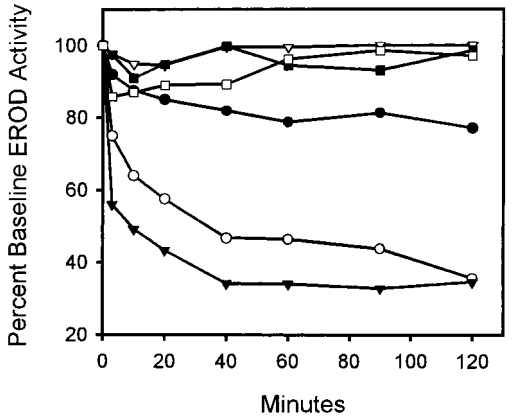

Figure 7. Time course of inhibition of EROD by thiol conjugates of PEITC, final assay concentration, 88 μM. (●) PEITC-*N*AC, (○) PEITC-GSH, (▼) PEITC-Cys, (▽) *N*AC, (□) GSH, (■) Cys.

2B1 and P450 1A1 are enhanced (Figures 6 and 7). The data indicate that, for each conjugate, both k_1 and $Dt_{1/2}$ are good predictors of relative inhibitory potency—the larger the absolute value of k_1 and the shorter the time

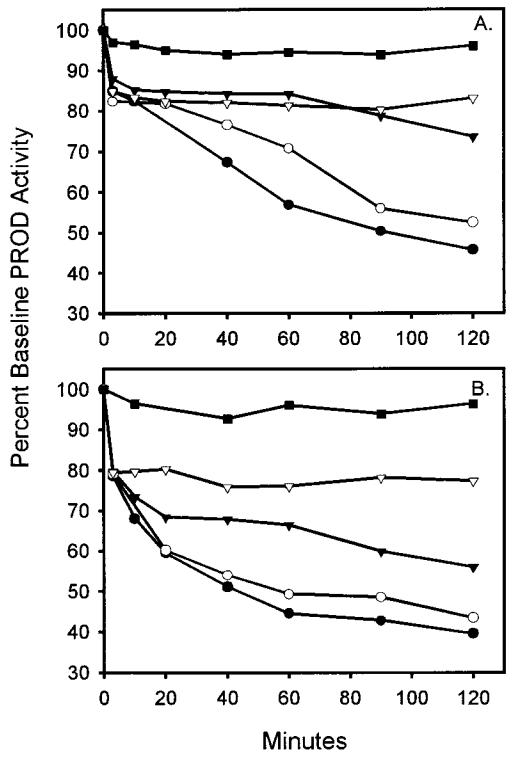

Figure 8. Inhibition of PROD by PEITC-NAC and PEITC-GSH. Assay concentration 4 μM. Effect of addition of GSH to incubation medium containing the respective thiol conjugate. (A) (●) 100 μM PEITC-*N*AC (control), (○) 100 μM PEITC-*N*AC + 100 μM GSH, (▼) 100 μM PEITC-*N*AC + 250 μM GSH, (▽) 100 μM PEITC-*N*AC + 1000 μM GSH, (■) 1000 μM GSH (no ITC-thiol). (B) (●) 100 μM PEITC-GSH (control), (○) 100 μM PEITC-GSH + 100 μM GSH, (▼) 100 μM PEITC-GSH + 250 μM GSH, (▽) 100 μM PEITC-GSH + 1000 μM GSH, (■) 1000 μM GSH (no ITC-thiol).

for $Dt_{1/2}$, the more potent a conjugate is as an inhibitor, and the shorter the corresponding $It_{1/2}$ (Table 1). Since Cys, GSH, and *N*AC have essentially no effect on the activity of P450 1A1 (Figure 7) or P450 2B1 (data not shown), it is concluded that free ITCs are predominantly responsible for the in vitro inhibition of CYP isozymes observed in this study. ITCs are potent inhibitors of P450 enzymes, as we have shown previously that 2 μM PEITC reacts very quickly (>75% inhibition in 15 s) to inhibit PROD (*18*). Furthermore, our data corroborate earlier results in which we found that the IC_{50}s of thiol conjugates of PEITC for PROD increased in the order PEITC-Cys (4.8 μM), PEITC-GSH (13.5 μM), and PEITC-*N*AC (30 μM). The IC_{50}s of thiol conjugates of PEITC were thus severalfold higher than the IC_{50} for PEITC (1.8 μM) (*18*). The IC_{50} values for thiol conjugates of SFN were not presented because of the unstable nature of the Cys conjugate, which had to be purified by HPLC after preparation, prior to conducting PROD inhibition studies. Sufficient amounts of pure Cys-SFN were not obtained.

The order of the half-life of deconjugation for all the ITC conjugates investigated was consistent for most ITCs studied, Cys < GSH < *N*AC, suggesting that the stability of thiol conjugates in aqueous solution is influenced greatly by the thiol moiety. The *N*AC conjugates were least easily deconjugated, indicating that the final products of the mercapturic acid pathway are more stable than their GSH and Cys conjugate precursors. The chain length of the various arylalkyl ITCs, especially for *N*AC conjugates, appeared to influence the rates of deconju-

gation as well (Table 1). It has been reported that increasing the carbon chain length decreases the rate of reaction of arylalkyl ITCs with GSH (*22*). The longer carbon chain lengths may provide an inductive effect, stabilizing the dithiocarbamate amino hydrogen from being attacked by OH^-. On that basis, increasing the number of alkyl carbons would tend to increase the stability of ITC-thiol conjugates. Further investigations will be required, however, to clearly determine the chemical factors that influence the rates of decomposition of ITC-thiol conjugates.

In an equilibrium process, products of deconjugation would be expected to slow the rate of cleavage of thiol conjugates, on the basis of the law of mass action, until a constant proportion of conjugate and products are formed. The shape of a representative decomposition curve for PEITC-GSH at pH 7.4 in Figure 3 demonstrates such an effect. Furthermore, the figure illustrates that even very low physiological concentrations of GSH (250 μM) markedly reduce the rate of decomposition of thiol conjugates of ITCs. Plasma concentrations of GSH are generally below 1 mM, while intracellular concentrations range from 0.5 to 10 mM (*26*, *27*). Thus, it would be expected that the plasma and tissue concentrations of GSH could retard the deconjugation of ITC-thiol conjugates in vivo, hence free ITCs would not be readily available from thiol conjugates.

The behavior of thiol conjugates of ITCs in tissues or cells was not examined, but an early study demonstrated that addition of GSH or Cys (5 mM) to the culture medium significantly reduced the toxicity of allyl isothiocyanate (AITC), BITC, and their respective GSH and Cys conjugates for RL-4 cells (*28*). More recently, Zhang reported that hydrolysis of BITC-GSH and SFN-GSH were required for their cellular uptake, and that only the free ITCs were transported through the cell membrane into the cytoplasm of murine hepatoma cells in culture. Addition of 0.05−5.0 mM GSH and other thiols to the culture medium strongly inhibited the cellular uptake of BITC-GSH and SFN-GSH (*29*).

Once having been absorbed through the cell membrane, intracellular ITCs are quite reactive, reacting or binding readily with P450 enzymes, sulfhydryl or amino groups of other proteins and amino acids, and GSH. The intracellular GSH content is rapidly depleted by spontaneous and enzymatic reactions with ITCs or other reactive chemicals, with concomitant formation of GSH-conjugates. GSH-ITCs and other GSH-conjugates are then transported out of the cell (*29*−*31*), possibly by the ATP-dependent action of membrane glycoproteins of the multidrug-resistance family (*32*).

The decomposition of thiol conjugates of ITCs was clearly pH dependent, at least for the two conjugates studied. A mechanism for the influence of hydroxyl ions on the decomposition of thiol conjugates of ITCs was proposed by others, in which a hydroxyl ion deprotonates the conjugate nitrogen, leading to elimination of the thiol leaving group and regeneration of the ITC (*33*). The physiological implication for the influence of pH on thiol-ITC decomposition is that orally administered thiol-ITCs would remain intact when absorbed through the stomach mucosa, but would probably dissociate to ITCs to a much greater extent in the intestinal tract (pH 5−8).

Early studies on the activity of ITCs reported that inhibition of P450 was a major mechanism for their chemopreventive effects (*2*, *3*, *15*), and it has been confirmed that inactivation of P450 2B1 and P450 2E1 by ITCs involves covalent binding and protein modification (*34*, *35*). Moreover, in addition to inhibition of P450, recent reports have shown that ITCs induce apoptosis by activation of pathways involving Ap-1 or NFκB (*10*, *36*), c-Jun-N-terminal kinase (*36*−*38*), various caspases (*13*, *39*, *40*), and p53 (*10*, *41*). The thiol conjugates of ITCs have been shown to inhibit cell division and promote apoptosis (*12*−*14*), which may be the mechanism whereby they exert chemopreventive activity at post-initiation stages of carcinogenesis (*9*−*11*). Thus, inhibition of tumorigenesis by blocking carcinogen activation by isozymes of P450 is apparently only one of the mechanisms of action of ITCs and their conjugates. We conclude that these recently published reports suggest that blocking carcinogen activation by P450 may be considerably less critical than previously thought. This study would corroborate such a conclusion regarding the role of P450 inhibition by thiol-ITCs, especially when sufficient concentrations of GSH are readily available in the cells of tissues and plasma to retard their decomposition to ITCs. We are conducting experiments on the uptake of dual-labeled [^{3}H]PEITC-[^{14}C]-*N*AC by cultured cells and in animals to further investigate the relationship between extracellular thiols and the decomposition and uptake of ITC conjugates.

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