

Identification and Quantification of the *N*-Acetylcysteine Conjugate of Allyl Isothiocyanate in Human Urine after Ingestion of Mustard¹

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Abstract

Allyl isothiocyanate (AITC) is a constituent of cruciferous vegetables. It occurs widely in the human diet as a natural ingredient or food additive. AITC possesses numerous biochemical and physiological activities. It is cytotoxic and tumorigenic at high doses and also is a modulator of enzymes involved in metabolism of xenobiotics, including carcinogens. It is plausible that the wide consumption of dietary AITC may have profound effects on human health. To facilitate investigations of the effects of dietary AITC in humans, a method of measuring its uptake is needed. In this study, a urinary marker was developed for quantifying AITC uptake in humans. Four adult volunteers were asked to eat a meal containing brown mustard as the source of AITC. The 48-h urine samples were collected from these individuals and analyzed by reverse phase high performance liquid chromatography. A major urinary metabolite was found, which was identified as *N*-acetyl-*S*-(*N*-allylthiocarbamoyl)-*L*-cysteine, the *N*-acetylcysteine conjugate of AITC, by comparing its retention time and UV, nuclear magnetic resonance, and mass spectra with those of the synthetic standard. After ingestion of mustard, the AITC conjugate was detected in urine collected from 0 to 12 h. No conjugate was found in urine samples collected after 12 h. The major portion of this metabolite was excreted within 8 h. The average total excretion of AITC conjugate was 5.4 ± 1.7 (SD) mg after consumption of 10 g of mustard and 12.8 ± 2.0 mg when 20 g of mustard was consumed. Thus, a dose-dependent excretion of this metabolite was demonstrated. The average conversion rate of AITC to its urinary *N*-acetylcysteine conjugate in humans was estimated to be $53.5 \pm 8.1\%$. These results suggest that the urinary *N*-acetylcysteine conjugate of AITC may be a convenient

and useful biomarker for quantifying human exposure to AITC.

Introduction

AITC³ is widely present in cruciferous vegetables such as cabbage, broccoli, kale, cauliflower, and horseradish (1–3). It is also commonly used in the human diet as a flavor agent (4). Like other isothiocyanates, AITC inhibits microsomal enzyme activities (5). Previous studies have shown that liver microsomes, obtained from rats that were fed a diet containing AITC, metabolize nitrosamines to a lesser extent than those of the untreated rats (6). AITC and its glucosinolate precursor, sinigrin, given in the diet, also inhibit hepatic DNA methylation induced by the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in rats (6–8). These results suggest the potential of AITC in modulating the carcinogenic activities of nitrosamines, since many arylalkyl isothiocyanates structurally related to AITC are known to be inhibitors of lung tumorigenesis induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (9). It was shown recently that AITC inhibits the growth of human cancer cells *in vitro* (10). Furthermore, several authors have reported that AITC induces the Phase II detoxification enzyme glutathione *S*-transferases (11, 12). On the other hand, chronic treatment with high doses of AITC induces urinary bladder tumors in rats (13). The diverse biochemical and biological activities of AITC and its wide consumption suggest its potential effects on human health.

Human exposure to AITC is mainly through the consumption of mustard, in particular brown mustard, and cruciferous vegetables. Because information on the exact content of AITC in these foods usually is not available and sometimes is impossible to obtain due to different storage and cultivation conditions, it is difficult to estimate the uptake of AITC in humans (4). Therefore, a marker would be useful to quantitatively monitor human exposure to AITC through diet. This information will be used to evaluate in epidemiological investigations the possible effects of dietary AITC on human health. Previously, it has been shown that the *N*-acetylcysteine conjugate of AITC (Fig. 1) is a urinary metabolite in rodents treated with AITC (14, 15). The urinary metabolites of the structural analogues of AITC, BITC and PEITC, have been studied in humans (16, 17). In this study, we describe the identification and use of the *N*-acetylcysteine conjugate of AITC as a simple and con-

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³ The abbreviations used are: AITC, allyl isothiocyanate; BITC, benzyl isothiocyanate; PEITC, phenethyl isothiocyanate; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; s, singlet; d, doublet; t, triplet; dd, doublet of doublets; ddd, doublet of doublets of doublets; ddt, doublet of doublets of triplets.

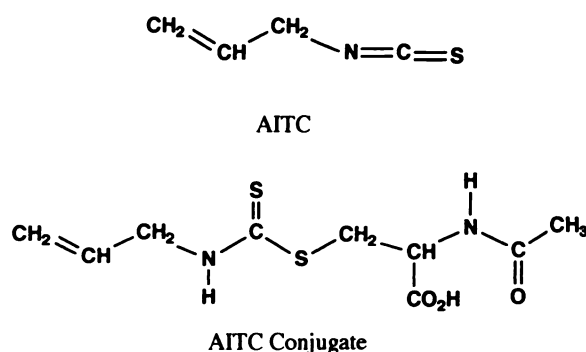


Fig. 1. Structures of AITC and its *N*-acetylcysteine conjugate.

venient urinary marker for the uptake of AITC after a mustard meal.

Materials and Methods

Instrumentation. NMR spectra were recorded on a Bruker AM 360 WB spectrometer using methanol- d_4 as solvent. Negative ion desorption chemical ionization mass spectra were obtained on a Hewlett-Packard 5988A mass spectrometer. A HPLC system (Waters Associates, MA) equipped with an automatic gradient controller, two Model 501 pumps, and a Waters 990 photodiode array detector in conjunction with reverse-phase C_{18} columns were used in the analyses and purification of the *N*-acetylcysteine conjugate of AITC. A Varian 3400 gas chromatograph equipped with a fused silica capillary column (60 m x 0.32 mm inside diameter, 1 μ m thickness, DB-1; J & W, Inc.) and a flame ionization detector were used to analyze the concentration of AITC in the mustard paste.

Chemicals. AITC was purchased from Aldrich Chemical Co. (Milwaukee, WI) and *N*-acetylcysteine was purchased from Sigma Chemical Co. (St. Louis, MO). The *N*-acetylcysteine conjugate of AITC was prepared as described in the literature (18) and was characterized by ^1H , ^{13}C -NMR spectroscopy, and mass spectrometry. The measured chemical shifts (δ) and coupling constants (J) are given as: ^1H -NMR (360 MHz, in CD_3OD , δ in ppm referenced to tetramethylsilane, 2.00 (3H, s, CH_3), 3.55 (1H, dd, J : 14.1, 8.6 Hz, Cys- CH_2), 4.02 (1H, dd, J : 14.1, 4.7 Hz, Cys- CH_2), 4.28–4.40 (2H, ddd, J : 12.2, 5.6, 1.5 Hz, allyl- CH_2), 4.72 (1H, dd, J : 8.6, 4.7 Hz, Cys-CH), 5.18 (1H, ddd, J : 10.2, 1.5, 1.5 Hz, *cis*-vinyl- CH_2), 5.26 (1H, ddd, J : 17.2, 1.5, 1.5 Hz, *trans*-vinyl- CH_2), 5.94 (1H, ddt, 17.2, 10.3, 5.7 Hz, vinyl-CH); ^{13}C -NMR (92.52 MHz, in CD_3OD , δ in ppm referenced to tetramethylsilane), 199.2 ($\text{C}=\text{S}$), 173.5, 173.2 (two $\text{C}=\text{O}$), 133.7 ($\text{C}=\text{C}$), 117.6 ($\text{CH}_2=\text{C}$), 53.8 (Cys- CH), 50.4 (allyl- CH_2), 38.3 (Cys- CH_2), 22.8 ($\text{N}-\text{CH}_3$); MS (m/e), 261 (M-H), 244, 221, 162, 131 (base peak), 58. Grey Poupon Dijon mustard was purchased from a local grocery store and kept refrigerated after opening. Mustard was chosen as a source of AITC because it is frequently used in cooking and thus it is relatively convenient to use in human studies.

Quantitative Analysis of AITC in Grey Poupon Country Dijon Mustard. Grey Poupon Dijon Mustard paste (100 g), combined with *tert*-butyl isothiocyanate (14.36 mg) as an internal standard, was thoroughly mixed with 1000 ml of distilled water and 200 g of NaCl. The mixture was stirred

with 200 ml of CH_2Cl_2 for 3 h and then filtered through Celite 545. After filtration, the CH_2Cl_2 phase was separated from the aqueous phase and subsequently dried over 20 g of anhydrous Na_2SO_4 . After removing Na_2SO_4 by filtration, the CH_2Cl_2 extract was concentrated by a stream of N_2 gas. The concentrated extract was used in the gas chromatography analysis using the following conditions: injector temperature, 270°C, detector temperature, 300°C; helium carrier flow rate, 1 ml/min; temperature program, 40°C (5 min), 2°C/min, 260°C (20 min); split ratio, 50:1.

Human Studies. Two experiments using different amounts of mustard were performed. Each experiment involved four adult volunteers (two males and two females, age 20–45). In the first experiment, 10 g of mustard was ingested with bagel or bread at breakfast by each participant. All participants were advised to avoid cruciferous vegetables, mustard, and mustard flavored foods in the diet 2 days prior to and during the experiment. In the control experiment, all participants were asked to eat the same food as in the experimental diet with the only exception of mustard. In the second experiment, participant 1 in the first experiment was replaced by another volunteer of the same sex. The same protocol was used except that 20 g of mustard was consumed with turkey or chicken sandwiches in a lunch. In both experiments, urine samples were collected at intervals of 0–2, 2–4, 4–8, 8–12, 12–24, 24–36, and 36–48 h following breakfast or lunch. Urine samples were analyzed immediately or stored at -20°C overnight. After thawing, an aliquot (50 μ l) of clear urine sample (the sample was centrifuged if not clear) was analyzed by a reverse phase HPLC system consisting of a Waters C_{18} - μ Bondapak column eluted isocratically with acetonitrile (10%) in 20 mM phosphate buffer (pH 3.0) at a flow rate of 1 ml/min.

Quantification. The HPLC peak of AITC conjugate detected at wavelength of 254 nm was used for integration. Standard solutions were prepared in 20 mM phosphate buffer (pH 3.0) with various concentrations of a synthetic *N*-acetylcysteine conjugate of AITC. The urinary metabolite was quantified with a calibration curve obtained using these standard solutions, which is linear over the concentration range examined (10^{-6} to 10^{-4} M). The urine samples were analyzed in the same fashion as the standards. Single and triple HPLC measurements were performed for samples obtained from experiments 1 and 2, respectively.

Isolation and Identification of the *N*-Acetylcysteine Conjugate of AITC in Human Urine. All crude urine samples collected in experiment 1 from 2–4 h following ingestion of mustard were combined (800 ml). Ammonium sulfate (160 g) was added and dissolved in the urine. The pH of the solution was adjusted to 3 with 12 N HCl. The acidic medium prevents possible decomposition of the conjugate during the work-up process. The solution was extracted with ethyl acetate (2×200 ml). The organic phase was washed twice with water (100 and 40 ml) and once with saturated NaCl solution (40 ml). After removing the solvent by a rotary evaporator under vacuum, the solid residue was dissolved in 5 ml of deionized water. Using a semipreparative reverse-phase C_{18} HPLC column (Whatman Partisil 10 ODS-3 column Magnum 9), a mobile phase of 20% acetonitrile in 20 mM aqueous phosphate buffer (pH 3.0), and an isocratic elution at a flow rate of 2.5 ml/min, the AITC conjugate eluted at 30 min was purified and obtained in sufficient quantity after repetitive runs. The collected fractions were combined and evaporated to dryness under vac-

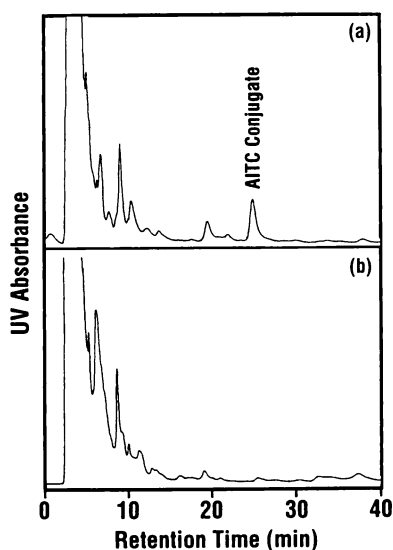


Fig. 2. Reverse phase HPLC chromatograms obtained from analysis of human urine after the mustard ingestion (a) and before the mustard ingestion (b).

uum. The residue was extracted with methanol. The extracts were combined and the solvent was removed in a vacuum to afford a solid. The compound was identified as the *N*-acetylcysteine conjugate of AITC by comparing its retention time, UV, $^1\text{H-NMR}$, and mass spectra with those of the synthetic conjugate.

Results

Two separate experiments were performed to establish the uptake-dependent excretion of the AITC conjugate in humans. In each experiment, a major metabolite of AITC was identified in the urine collected after ingestion of mustard and the metabolite was not present in the urine after consuming the control diet (Fig. 2). The peak at 25.2 min coelutes with the synthetic standard of the *N*-acetylcysteine conjugate. This compound was isolated and purified from crude urine samples as described in "Materials and Methods." It has the same characteristic UV absorptions as the synthetic standard (Fig. 3). Its identity was further confirmed to be *N*-acetyl-S-(*N*-allylthiocarbamoyl)-L-cysteine by comparing its $^1\text{H-NMR}$ and mass spectra with those of the synthetic standard (Fig. 4).

In both experiments, the *N*-acetylcysteine conjugate of AITC was detected in all urine samples collected within 12 h after ingestion of mustard. The detection limit using this method is in the 1–10 ng range. No metabolite was detected in the urine after 12 h by using the direct measurement described here or by using organic solvent extraction procedures reported previously (16). The cumulative amounts of the AITC conjugate in the urine collected at different time intervals are shown in Table 1. Normally, the amount of excreted AITC conjugate reaches the maximum between 2 and 8 h. The amount of excretion depends on both the concentration of AITC conjugate in urine and the volume of urine collected in a given time interval. The maximum concentration of AITC conjugate excreted in urine was observed at 2–4 h following ingestion of mustard, as shown in Fig. 5. The majority of the conjugate was excreted within 8 h. The total average excretion is proportional to the

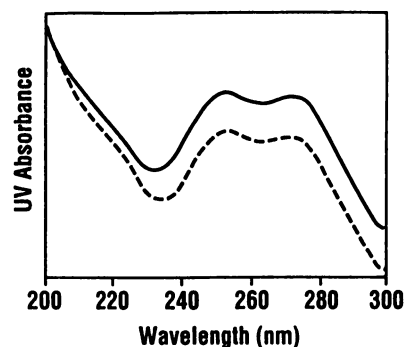


Fig. 3. Comparison of UV spectra of the synthetic AITC conjugate (—) and the urinary metabolite (---).

amount of mustard consumed, *i.e.*, 5.4 ± 1.7 (SD) mg (3.6–7.6 mg) and 12.8 ± 2.0 mg (10.5–15.2 mg) are excreted corresponding to 10 and 20 g of mustard consumed. These results showed an uptake-dependent excretion of the AITC metabolite after mustard meals.

Consistent with the literature report (18), we have found that the *N*-acetylcysteine conjugate of AITC is in equilibrium with its free form. In our study, the equilibrium was evident by the presence of a small peak eluting after the conjugate which coeluted with AITC under the HPLC conditions used. A significant percentage of the *N*-acetylcysteine conjugate of AITC decomposed during an extended period of storage in a neutral medium, even at -20°C . Because of its instability, caution should be taken in quantifying the levels of this conjugate in the urine. Previously, we have found that PEITC is stabilized in acidic medium (16); it is likely that the AITC conjugate would be considerably more stable at acidic pH.

Although brown mustard is known to be rich in AITC (4), the exact content of AITC in the commercial products was not available. We have used gas chromatography to quantitatively analyze AITC in the mustard paste used in the human experiments. The result showed that the AITC content of the mustard is 453 ppm, or 0.453 mg of AITC/g of mustard. Using this information, we were able to calculate the conversion rate of AITC to its *N*-acetylcysteine conjugate in humans, as shown in Table 2. The average of the individual conversion rates is $53.5 \pm 8.1\%$, which is consistent with the previous studies on the metabolism of BITC and PEITC in humans (16, 17). Those studies have shown that the conversion rates of these two isothiocyanates to their corresponding urinary *N*-acetylcysteine conjugates are $53.7 \pm 5.9\%$ and $47 \pm 16\%$, respectively. Assuming that the average conversion rate of AITC obtained here is applicable to a larger population and is independent of the source of AITC, one may estimate the amount of AITC to which humans were recently exposed through the consumption of various foods and vegetables by simply measuring their urinary excretions of the *N*-acetylcysteine conjugate of AITC.

Discussion

The *in vivo* metabolism of several natural isothiocyanates has been studied in rodents and humans. For instance, the *N*-acetylcysteine conjugates of AITC and PEITC are excreted in the urine of mice (14, 19), although the major

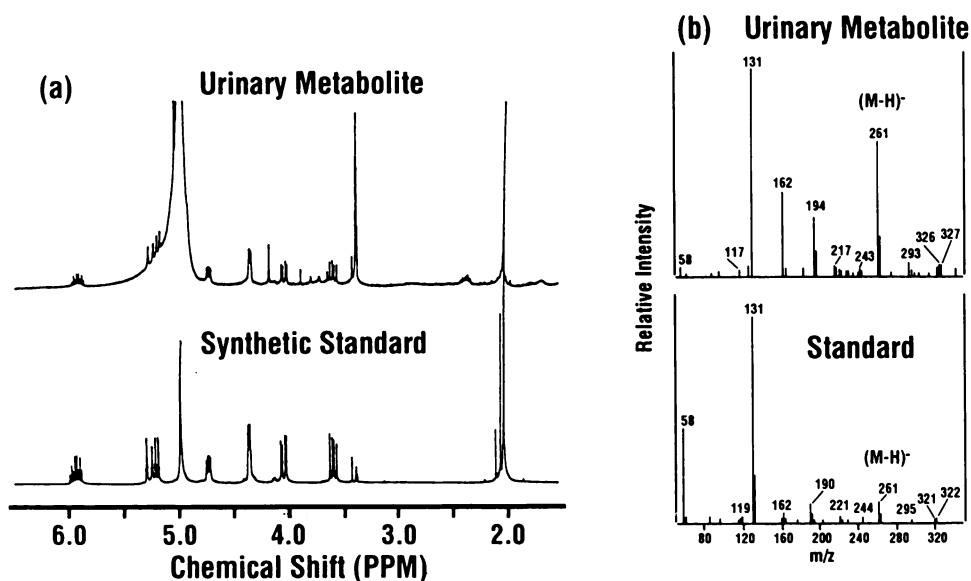


Fig. 4. (a) Comparison of the 360 MHz $^1\text{H-NMR}$ of the synthetic standard of the *N*-acetylcysteine conjugate of AITC and the metabolite isolated from human urine samples after ingestion of mustard. Note that differences for solvent peak intensities at 3.35 and 4.97 ppm are due to the different concentrations of the two samples. The resonances for the AITC conjugate are identical in two spectra. (b) Comparison of their mass spectra. The major fragments $\text{C}_4\text{H}_5\text{NS}_2$ (131 m/e) and $\text{C}_2\text{H}_4\text{NO}$ (58 m/e) for the AITC conjugate negative ion (261 m/e) were observed in both spectra.

Table 1 Cumulative amounts of the *N*-acetylcysteine conjugate of AITC in human urine 12 h after ingestion of mustard

Subject	Time interval (h)	Experiment 1		Experiment 2	
		AITC conjugate ^a (mg)	Total excretion (mg)	AITC conjugate ^b (mg)	Total excretion (mg)
1 ^c	0–2	0.7		2.8 ± 0.1	
	2–4	2.6		4.0 ± 0.2	
	4–8	2.4		3.2 ± 0.1	
	8–12	1.6	7.6	0.5 ± 0.1	10.5
2	0–2	2.6		4.7 ± 0.1	
	2–4	1.1		^d	
	4–8	ND ^e		5.0 ± 0.1	
	8–12	ND ^e	3.6	3.6 ± 0.1	13.3
3	0–2	3.6		2.8 ± 0.1	
	2–4	^d		6.1 ± 0.5	
	4–8	1.2		2.9 ± 0.2	
	8–12	0.4	5.3	0.5 ± 0.0	12.3
4	0–2	1.1		1.9 ± 0.2	
	2–4	1.2		5.4 ± 0.1	
	4–8	2.2		6.2 ± 0.6	
	8–12	0.4	4.9	1.7 ± 0.2	15.2

^a Based on one measurement for each sample.

^b Mean ± SD of three separate determinations.

^c Subject 1 participated in experiment 1 but was replaced by another individual of the same sex in experiment 2.

^d No urine was excreted during this period.

^e ND, not determined due to peak overlap.

metabolite of PEITC in mice is a cyclic mercaptopyruvic conjugate (19). However, the *N*-acetylcysteine conjugates are the major urinary metabolites in rats treated with AITC and BITC (15, 20). In humans, the *N*-acetylcysteine conjugates of BITC and PEITC appear to be the only urinary metabolites following ingestion of BITC, gardencress, and watercress (16, 17). However, to the best of our knowledge, the metabolism of AITC in humans has not been reported before.

Conjugations of isothiocyanates with glutathione appear to be the major metabolic pathway in humans, since most of their urinary metabolites are mercapturic acids or other derivatives from glutathione conjugates (14–19). Although the Phase II enzyme glutathione *S*-transferase-catalyzed conjugation of isothiocyanates is considered to be a natural detoxification process (16, 17), it has been postulated that this pathway may also be involved in the cyto-

Fig. 5. The concentration of the *N*-acetylcysteine conjugate of AITC in urine collected from four volunteers versus time (hours) following the ingestion of mustard in experiment 2.

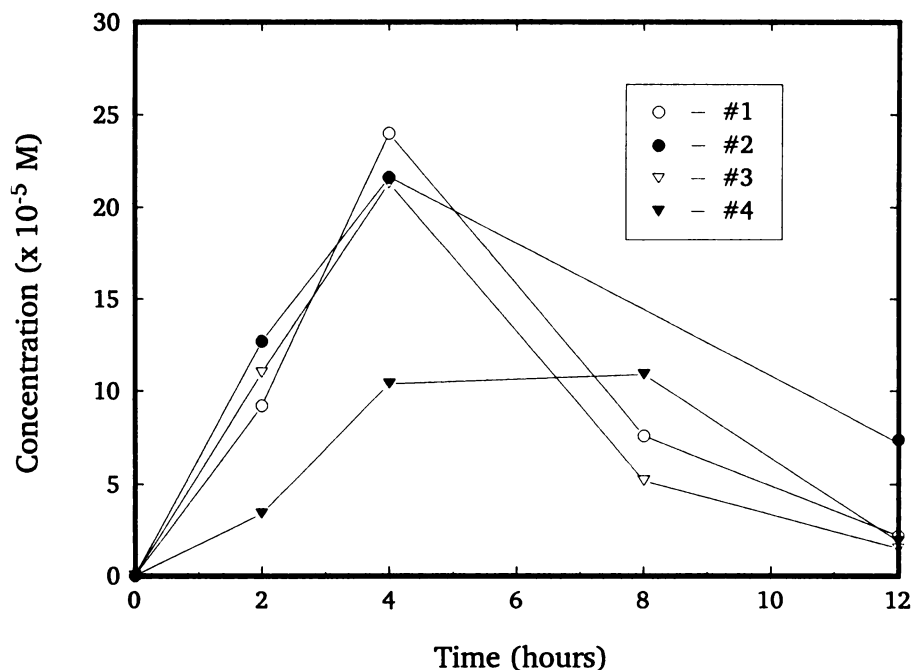


Table 2 Estimated percentage of conversion of allyl isothiocyanate to the *N*-acetylcysteine conjugate of AITC in human after a mustard meal^a

Subject	Total conjugate excreted (mg)	AITC equivalent (mg)	Conversion (%)
1	10.5	4.0	44.2
2	13.3	5.0	55.2
3	12.3	4.6	50.8
4	15.2	5.7	63.4

^a Based on 0.453 mg/g weight of AITC in Grey Poupon mustard consumed in experiment 2.

toxicity of isothiocyanates (18). The glutathione conjugates of isothiocyanates are usually subject to further degradation to give final metabolites, the *N*-acetylcysteine conjugates of isothiocyanates, by enzymes such as γ -glutamyltranspeptidase, cysteinylglycine, and *N*-acetyltransferase (20). Recently, the activities of the detoxification enzyme glutathione *S*-transferase have been associated with the risk of certain human cancers (21, 22). A survey of smokers demonstrated that individuals lacking glutathione *S*-transferase μ had a significantly higher incidence of lung cancer than those who display glutathione *S*-transferase μ activity (23). A discrepancy between phenotyping and genotyping the isozymes of glutathione *S*-transferase in relation to the risk of lung cancer in smokers was also reported (24, 25). The levels of excretion of the AITC conjugate in the urine following mustard consumption may be used to phenotype an individual for the activity of these enzymes. Therefore, it would be important to identify the specific glutathione transferase isozymes responsible for the conjugation of AITC.

It has been well documented that compounds in cruciferous vegetables induce Phase II detoxification enzymes, such as quinone reductase and glutathione *S*-transferase (11, 26). An isothiocyanate isolated from broccoli, (–)-1-isothiocyanato-(4*R*)-(methylsulfinyl)butane (CH₃-SO-(CH₂)₄-NCS, sulforaphane), was shown to be a strong Phase

II enzyme inducer (27). Knowing that consumption of vegetables reduces the risk of cancer (28, 29), it is noteworthy that these isothiocyanates isolated from natural sources, including AITC, PEITC, and sulforaphane, may function as either Phase I enzyme inhibitors (5, 30, 31), which prevent the activation of carcinogens, and/or as Phase II enzyme inducers (11, 27). Much work is needed to further establish the detailed mechanism regarding how these naturally occurring compounds may work in humans to reduce the risk of cancer. The results presented here should provide a useful tool in the epidemiological investigations of the biological role of AITC in humans.

Acknowledgments

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