Effects of Human and Rat Glutathione S-Transferases on the Covalent DNA Binding of the N-Acetoxy Derivatives of Heterocyclic Amine Carcinogens in Vitro: A Possible Mechanism of Organ Specificity in Their Carcinogenesis

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ABSTRACT

The effects of glutathione (GSH) and of purified human and rat GSH S-transferases (GSTs) on the covalent DNA binding of 3 putative ultimate food-borne carcinogens, the N-acetoxy derivatives of 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP), 2-amino-3-methylimidazo(4,5-f)quinoline (IQ), and 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx), were studied in vitro. GSH (5 mM) alone slightly inhibited (10%) the DNA binding of N-acetoxy-PhIP (100 μM) at pH 7.5, but the binding could be strongly inhibited in the presence of both GSH and GSTs. Among human GSTs, the isozyme A1-1 (α-class) was most effective (90% inhibition) followed by A1-2 (40% inhibition); the effect of adding A2-2 was negligible, suggesting that the activity exists in subunit A1. In addition, human GST P1-1 (π-class) also had some inhibitory effect (30%). Among the rat GSTs tested, GST 1-2 and GST 12-12 (9-class), which are the equivalent of human A1-2 and T2-2, respectively, were able to inhibit DNA binding of N-acetoxy-PhIP (75 and 40%, respectively). This activity toward N-acetoxy-PhIP was dependent on enzyme concentration and was subject to inactivation by triethyltin bromide, a known GST inhibitor. In contrast, the binding of N-acetoxy-IQ or N-acetoxy-MeIQx to DNA was unaffected by addition of the human or rat GSTs; however, GSH alone significantly inhibited (40%) their binding to DNA. High-performance liquid chromatographic analyses of incubation mixtures containing N-acetoxy-PhIP, GSH, and GST A1-1 failed to detect GSH conjugates of PhIP. Only oxidized glutathione and the parent amine, PhIP, were detected as reaction products, suggesting a redox mechanism.

INTRODUCTION

Heterocyclic amines formed in protein-rich foods during cooking and heating processes are potent mutagens, and the majority have been established as carcinogens in experimental animals (1, 2). Humans are believed to be exposed to these mutagens daily in their diet; thus, significant risk for the development of human cancers is of major concern. The biological effects of heterocyclic amines, like most chemical carcinogens, require prior enzymatic activation. Oxidation of the exocyclic amino group to form the corresponding N-hydroxy derivative is catalyzed mainly by cytochrome P-450IA2 and is believed to be the initial activation step (3–7). The N-hydroxy derivatives are then further activated by O-acetylation or O-sulfonation to form highly reactive N-acetoxy or N-sulfonyl derivatives that are likely to be ultimate carcinogens producing DNA damage and consequently initiating the neoplastic process (8–12). The liver is the most active organ in metabolizing heterocyclic amines into electrophiles capable of forming carcinogen-DNA adducts; however, this activity has also been shown to exist in extrahepatic tissues such as the small intestine, colon, lung, and mammary tissue (9, 13–15). The role of O-acetylation in the carcinogenesis of heterocyclic amines appears to be significant, since the rapid acetylator/rapid P-450IA2 phenotype is correlated with increased colorectal cancer susceptibility (16–18).

Most heterocyclic amines are multiorgan carcinogens in rodents, with the liver being a common target organ (1). IQ2 and MeIQx, for example, were shown to be hepatocarcinogens in rodents or monkeys (2, 19–22). PhIP, however, did not induce liver cancer when fed to adult rats or mice. Instead, it induced colon and mammary cancers in rats and lymphomas in mice (23, 24). Studies on DNA adduct formation in vivo in rats also revealed that, in contrast to IQ and MeIQx, which formed high adduct levels in the liver (25–27), PhIP produced high adduct levels in extrahepatic organs such as the pancreas, heart, and large intestines, but extremely low levels in the liver (25, 28–30). This differential organ specificity of genotoxicity and carcinogenesis suggests that the liver may have additional detoxifying pathways to prevent the carcinogenicity of PhIP.

It has been proposed that the following biotransformation pathways are detoxification mechanisms for some heterocyclic amines: N-glucuronidation and N-sulfation of parent compounds; and ring-hydroxylation followed by sulfate or glucuronosyl conjugations (6, 30–35). For PhIP, hepatic glucuronidation of the proximate carcinogen, N-hydroxy-PhIP, can also occur (30, 36). GSH and GSTs play an important role in the detoxification of many xenobiotics (37); however, their reported effects on heterocyclic amines appear to be inconsistent. It has been shown that the mutagenicity of 2-amino-1-methyl-5H-pyrido[4,3-b]indole and its N-oxidized derivative is enhanced by GSH in the presence of rat liver S9 or GST, due to the formation of a more reactive GSH conjugate (38, 39). In contrast, GSH had no effect on the mutagenicity of 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole, a methylated analogue of 2-amino-1-methyl-5H-pyrido[4,3-b]indole (39). Furthermore, it was reported that GSH and other thiols stimulated AcCoA-dependent binding of 2-hydroxymethylpyrido[1,2-a:3',2'-d]imidazole to DNA (40). However, it has been shown that the mutagenicity of 2-amino-1-methyl-5H-pyrido[4,3-b]indole and its N-oxidized derivative is enhanced by GSH in the presence of rat liver S9 or GST, due to the formation of a more reactive GSH conjugate (38, 39). In contrast, GSH had no effect on the mutagenicity of 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole, a methylated analogue of 2-amino-1-methyl-5H-pyrido[4,3-b]indole (39). Furthermore, it was reported that GSH and other thiols stimulated AcCoA-dependent binding of 2-hydroxymethylpyrido[1,2-a:3',2'-d]imidazole to DNA (40). However, the costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
GSH did have a protective role against IQ and PhIP damage in hepatocytes and in vivo in rat liver since depletion of cellular GSH resulted in elevated levels of IQ and PhIP metabolites covalently bound to DNA and an increase in PhIP-induced unscheduled DNA synthesis (41–43). These results indicated that the chemical properties of the reactive metabolites derived from different heterocyclic amines may be a critical determinant in their ability to undergo nonenzymatic reactions with GSH and/or reactions catalyzed by GSTs.

Several GST isozymes with differential substrate specificity are known to be present in liver cytosol (37). While the protective effect of GSH/GSTs against the genotoxicity of some heterocyclic amines has been documented (vide supra), it has not yet been determined which isozyme is involved in these reactions. Furthermore, little is known about the detoxifying effect of human GSTs on this class of carcinogens. In the present study, we have investigated the effect of purified human and rat GST isozymes on the detoxification of 3 N-acetoxy heterocyclic amines, i.e., IQ, MelIQx, and PhIP, using covalent DNA binding as an endpoint of genotoxicity. Since the N-acetoxy derivatives of these compounds are believed to be candidate ultimate carcinogens that produce DNA damage, they were therefore used as substrates. The activity of GSTs toward these carcinogens in cytosols prepared from human livers and colon mucosa was also studied.

MATERIALS AND METHODS

Chemicals. [Ring-3H]2-nitro-3-methylimidazo(4,5-f)quinoline, [ring-3H]2-nitro-3,8-dimethylimidazo(4,5-f)quinoxaline (86.3 mCi/mm), PhIP (ring-3H; 13.35 Ci/mmol), and unlabeled PhIP were purchased from Chemsyn Science Laboratories (Lenexa, KS) and Toronto Research Chemicals (Toronto, Ontario, Canada). [Ring-3H]2-nitro-3-methylimidazo(4,5-f)quinoline, [ring-3H]2-nitro-3,8-dimethylimidazo(4,5-f)quinoxaline, and [3H]PhIP (diluted to 12 mCi/mm) were further purified by silica gel column chromatography and by HPLC before use. The radiochemical purity was shown to be >99%, respectively. Cal F thymus DNA, AcCoA, reduced GSH, GSSG, GSH reductase, and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). TETB was from Alfa Products (Danvers, MA).

Synthesis. The nitro-derivatives of [3H]PhIP were synthesized as described previously (12). N-Hydroxy derivatives of IQ and MelIQs were prepared by reduction of the corresponding nitro analogues with ascorbic acid (9). N-Hydroxy-PhIP was obtained by reduction of nitro-PhIP with hydrazine and palladium on charcoal on (12). The N-hydroxy products were each dissolved in dimethyl sulfoxide:ethanol (4:1) under argon and stored in liquid nitrogen until use (within 2–3 months). The purity of each product was judged to be >95% by HPLC. N-Acetoxy-PhIP was prepared immediately prior to the enzyme assays by the addition of acetic anhydride (40-fold m excess) to an N-hydroxy-PhIP solution at 0°C with stirring as reported previously (12). N-Acetoxy-IQ and N-acetoxy-MelIQs were extremely unstable and were synthesized in situ in the assay medium as described below.

Preparation of Human and Rat GST Isozymes. Human GSTs A1-1, A1-2, A2-2, and M1a-1a were purified from liver cytosol (livers HL 133 and 135, kindly provided by F. P. Guengerich, Vanderbilt University, Nashville, TN) by sequential GSH-agarose affinity, hydroxyapatite adsorption, and ion-exchange fast protein liquid chromatography (Mono Q) chromatography as described by Raney et al. (44). Human GST P1-1 was similarly purified from postmortem kidneys. Rat GST 1-2 (α-class) was purified from liver cytosol by GSH-agarose affinity chromatography followed by sucrose density gradient isoelectric focusing and hydroxyapatite chromatography (45). Rat α-class GSTs 5-5 and 12-12 were prepared as described by Meyer et al. (46). Reversed-phase HPLC showed that GST purity was >97% in each case (47).

Preparation of Human Liver and Colon Cytosols. Human liver and colon tissues were obtained from different individuals undergoing necessary surgical procedures at the John L. McClellan Memorial Veterans Administration Hospital and at the University Hospital of Arkansas, Little Rock, AR. The tissues were immediately placed in cold saline, and then frozen quickly in liquid nitrogen, and stored at −80°C until preparation of cytosols. Sections of the livers were thawed and washed at 4°C with phosphate-buffered saline. The livers were minced and homogenized in an ice-cold Teflon-glass homogenizer in 10 mm potassium phosphate buffer (pH 7.4) containing 1.4 mg ml−1 β-mercaptoethanol and 0.25 m sucrose. The colon mucosal cells were removed from the tissues using a spatula and were homogenized as described for the liver. The cytosol was obtained by centrifugation of the homogenate at 105,000 × g for 45 min and kept at −80°C until use. Protein concentration was determined by the method of Lowry et al. (48).

Enzyme Assays. Assays for the purified GST isozymes were conducted in 100 mM potassium phosphate buffer (pH 7.5), containing 0.1 mM EDTA, 2 mg/ml calf thymus DNA, 5 mM GSH, and various amounts of GST isozymes as indicated in the Table and Figure legends. [3H]N-Acetoxy-PhIP, dissolved in dimethyl sulfoxide:ethanol (4:1), was added to the incubation mixture at the final concentration of 100 μM. N-Acetoxy-IQ and N-acetoxy-MelIQs were formed in situ by adding the radiolabeled N-hydroxy derivatives to the incubation mixture at a final concentration of 100 μM, followed immediately by a 40-fold m excess of acetic anhydride (49, 50). Under these assay conditions (1–2% solvent and 4 mM acetic anhydride in the mixture), the GST activity was only inhibited about 15% as measured using 1-chloro-2,4-dinitrobenzene as substrate. Assays without GSH or GST were included as controls.

GST activities toward N-acetoxy-PhIP in human liver and colon mucosal cytosol were determined in incubation mixtures (1 ml) containing 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 2 mg cytotoxic protein, 2 mg calf thymus DNA, 3 mM GSH, 5 μM TETB (added in 5 μl methanol), and 100 μM substrate dissolved in 10 μl of dimethyl sulfoxide:ethanol (4:1).

Assays were also carried out in human hepatic cytosols to measure GST activities toward the N-acetoxy derivatives of the heterocyclic amines that were each prepared in situ from the corresponding N-hydroxy derivatives by AcCoA-dependent enzymatic O-acetylation, as reported previously (9). A 1-ml assay mixture consisted of 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 2 mg DNA, 3 mg cytotoxic protein, 1 mM AcCoA, and 100 μM radiolabeled N-hydroxy heterocyclic amines (added in 10 μl dimethyl sulfoxide:ethanol). GSH (3 mM) and/or TETB (5 μM) were included in the assays where GST activity was measured.

The nonenzymatic effect of various concentrations of GSH on the covalent DNA binding of the N-acetoxy derivatives of the heterocyclic amines was determined using incubation conditions identical to that described above but without the GSTs.

The assay mixtures were purged with argon and warmed at 37°C for 3 min before the addition of N-hydroxy- or N-acetoxy heterocyclic amines. Upon adding the substrate under argon, the mixtures were incubated at 37°C for 30 min, unless otherwise specified. The incubations were terminated by the mixing with 2 vol of water-saturated n-butanol. The modified DNA was then isolated by multiple solvent extractions and precipitations, and the extent of covalent binding was measured by liquid scintillation counting as described previously (9, 12).

Determination of PhIP and GSSG. The stoichiometry of PhIP and GSSG formation was determined in the enzyme-catalyzed reactions. The typical reaction mixture (0.5 ml) consisted of 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 5 mM GSH, 100 μM [3H]N-acetoxy-PhIP, and 0–120 μg/ml rat GST 1–2. The reactions were carried out for 30 min at 37°C. For analysis of PhIP, the reactions were terminated by extraction twice with 0.5 ml of chloroform. The extraction efficiency was >99% as determined by measuring the recovery of [3H]PhIP in the 2 phases. After removal of solvent, the residue was dissolved in 50 μl chloroform for HPLC analysis. HPLC separations were done using a Waters Associates (Milford, MA) HPLC system that included a Waters model 911 photodiode array detector and a Radiomatic series A-500 radiochromatography detector with Radiomatic Flo-Scint III as the scintillator. A Waters μBondapak C18 column (3.9 × 300 mm) was used with a mobile phase of 20 mM diethylamine acetate buffer (pH 6.2) (solvent A) and methanol (solvent B). The elution was carried out with a linear gradient from 20 to 100% solvent B over 30 min at a flow rate of 2 ml/min. Under these conditions, PhIP was eluted near 18 min. The formation of PhIP was confirmed by comparison of its HPLC retention time and its UV spectrum with that of an authentic standard, and its quantitation was based on the percentage of radioactivity eluting from the column.

GSSG formed in the reaction mixture was determined essentially as described (51). Briefly, after incubation, 0.4 ml of the mixture was transferred to a cuvette containing 0.6 ml of 0.1 mM potassium phosphate buffer (pH 7.0) and

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The detoxification of N-acetoxy-PhIP by GSH and GSTs as measured by the inhibition of its covalent modification of DNA is shown in Table 1. Of the major human hepatic GSTs tested, namely A1-1, A1-2, A2-2, and M1a-1a, only the first 2, containing GST subunit A1, had significant activity. GST P1-1, which occurs in many extrahepatic tissues and is often increased in malignancy (52), also showed activity but was less effective than GST A1-1. In contrast to its detoxification of N-acetoxy-PhIP, there was no effect of human GST A1-2 on DNA modification by N-acetoxy-IQ or N-acetoxy-MeIQx. Rat GST 1-2, which is a α-class GST comparable to human A1-2, was also found to be effective in the inhibition of DNA modification by N-acetoxy-PhIP (Table 2).

The role of the θ-class GST family in heterocyclic amine detoxification was also examined. Human GSTs T1-1 (46) and T2-2 (53) have been identified but were not available in quantity for these assays. Hence, their rat orthologues, GSTs 5-5 and 12-12, were studied. While GST 5-5 had no significant effect, GST 12-12 showed inhibition of covalent DNA binding, notably at low enzyme concentration (Table 2).

Since preliminary experiments indicated that N-acetoxy-IQ and N-acetoxy-MeIQx were too unstable to be isolated and used in enzymatic assays, we used a method for generation of these substrates in situ by reacting acetic anhydride with the corresponding N-hydroxy precursors, as preliminary experiments showed that DNA modification by N-acetoxy-IQ or N-acetoxy-MeIQx. Rat GST 1-2, which contains GST subunit Al, strongly inhibited when 5 μM TETB, a known GST inhibitor (54), was added to the incubation medium, DNA binding increased 31-fold for IQ and 170-fold for MeIQx (Tables 1 and 2), confirming the formation of the more reactive N-acetoxy derivatives in situ. However, no GST-dependent inhibition of DNA binding of N-acetoxy-IQ and N-acetoxy-MeIQx was detected (Tables 1 and 2), although it was noted that GSH alone significantly inhibited the binding of both IQ and MeIQx derivatives to DNA. While the spontaneous reaction of GSH with N-acetoxy-IQ or N-acetoxy-MeIQx cannot be ruled out, this inhibitory effect may in part be attributed to the nonenzymatic reaction of GSH with the N-hydroxy precursors, as preliminary experiments showed that DNA binding of N-hydroxy-IQ and N-hydroxy-MeIQx but not N-hydroxy-PhIP was significantly inhibited by GSH (data not shown).

The effects of enzyme concentrations, assay pH, incubation time, and enzyme inhibitors on covalent DNA modification by N-acetoxy-PhIP were then studied using human GST A1-1, and the results are shown in Fig. 1. The reaction of N-acetoxy-PhIP with DNA was completed over the 10-min period of incubation at 37°C (Fig. 1C). Increasing incubation time to 30 min did not affect binding levels either in the absence or in the presence of GSH and/or GST A1-1. Carcinogen-DNA binding was decreased proportionately with the amount of enzyme added (Fig. 1A) and the enzyme activity was strongly inhibited when 5 μM TETB, a known GST inhibitor (54), was added to the reaction mixture (Fig. 1D). The optimal pH for the enzyme was pH 7.0–8.0 (Fig. 1B). Similar results were obtained using the rat GST 1-2 isozyme (data not shown). These data clearly demonstrated that N-acetoxy-PhIP is a substrate for human GST A1 and rat GST subunits 1 or 2.
In order to approach the *in vivo* situation, the effect of GST activity of human liver cytosol on the acetyltransferase-mediated DNA binding of the 3 N-hydroxy heterocyclic amines was investigated. In the incubations containing AcCoA and cytosolic acetyltransferase, the extent of the covalent DNA binding of the N-hydroxy derivatives of the 3 carcinogens was greatly increased as compared to that obtained upon incubation without the cofactor (Fig. 2). These results were consistent with that reported previously (9) and confirmed the role of human hepatic acetyltransferase in the activation of these N-hydroxy heterocyclic amines. Addition of 3 mM GSH to the cytosol resulted in 60% inhibition of acetyltransferase-mediated covalent binding of N-hydroxy-PhIP to DNA (80.3 versus 199.5 pmol/mg DNA; *P < 0.01*), suggesting detoxification of the ultimate carcinogen by GST. This effect was further demonstrated by the fact that the covalent DNA binding was significantly elevated by 175% (80.3—140 pmol/mg DNA; *P < 0.01*) when the GST inhibitor TETB was included in the incubation system (Fig. 2). However, in contrast to the results obtained with N-hydroxy-PhIP, the GST activity in the hepatic cytosol toward acetyltransferase-activated N-hydroxy-IQ or N-hydroxy-MeIQx was not detectable, although the DNA binding was slightly inhibited by the inclusion of GSH itself (Fig. 2). These results are in further agreement with those obtained using various purified human hepatic GSTs (Table 1).

The GST activities in 8 human hepatic cytosols and in 6 human colon mucosal cytosols were then measured using N-acetoxy-PhIP as substrate and covalent DNA binding in the presence and absence of TETB as an indicator of GST catalysis. The results shown in Fig. 3 clearly demonstrated that GST activity toward N-acetoxy-PhIP was expressed in all human livers, with individual variations of the activity being observed. The activity among the 8 individuals differed from 20 to 56%, expressed as relative inhibition of DNA binding measured with and without TETB (Fig. 3). Under the same assay conditions, however, no activity was detected for the GST-dependent detoxification of N-acetoxy-PhIP in the 6 human colon mucosal cytosols examined (Fig. 4).

HPLC analysis of the enzymatic reaction mixture failed to find GSH conjugates of PhIP or its derivatives, and PhIP appeared to be the only detectable heterocyclic amine reaction product. As shown in Fig. 5C in the incubation mixture containing 120 µg/ml of rat GST 1-2 isozyme, ~82% of N-acetoxy-PhIP was reductively converted to PhIP, while without added GST but with GSH, most of N-acetoxy-PhIP decomposed to an unknown product that had a retention time of about 25 min, with only a small portion being reduced back to PhIP (Fig. 5B). It was noticed that a small portion of N-acetoxy-PhIP was spontaneously converted to PhIP (Fig. 1A). The rate of reduction of N-acetoxy-PhIP to PhIP was increased with the amount of the GST (Table 3). These data suggested that an enzymatic redox process might be involved in the reaction mechanism. The formation of GSSG in the reaction mixture was therefore quantified and the results are presented in Table 3. As with the formation of PhIP, the formation of GSSG was enzyme-dependent. The ratio of GSSG to PhIP was found to be approximately 0.5 and was constant in all assays over the range of enzyme concentrations used (Table 3).

**DISCUSSION**

The process of chemical carcinogenesis is believed to be initiated by the covalent modification of DNA by electrophiles derived from carcinogen metabolism. Since GSTs catalyze the reaction of nucleophilic GSH with electrophilic metabolites of carcinogens (reviewed in Ref. 37), these enzymes can act to prevent initiation of chemical...
carcinogenesis by detoxifying electrophilic proximate and/or ultimate carcinogens. Detoxification by GSTs of the putative ultimate carcinogens, the N-acetoxy derivatives of the heterocyclic amines IQ, MeIQx, and PhIP, was therefore studied in vitro using covalent DNA modification as a measure of genotoxicity. Our results clearly demonstrated that certain human and rat GSTs are able to prevent the binding of N-acetoxy-PhIP to DNA. Human GST A1-1, A1-2, and rat GST 1-2 and 12-12 were found to be most active toward this carcinogen. However, these enzymes had no inhibitory effect on the DNA binding of N-acetoxy-IQ and N-acetoxy-MeIQx. While most heterocyclic amines including IQ and MeIQx formed high levels of DNA adducts in the liver and also induced liver cancers in experimental animals (1, 2, 25–27), PhIP is not hepatocarcinogenic (23, 29, 30), except in the neonatal mouse (55). The mechanism of this organotropism for the genotoxicity and carcinogenicity of these heterocyclic amines is not yet clear; yet the balance of metabolic activation and inactivation of the carcinogens in the liver is likely to be one of the determinants. Previous studies have shown that PhIP, IQ, and MeIQx were readily activated by rat and human liver microsomes (9, 56) and by O-acetyltransferase (9). The substrate specificity and activation rates for PhIP and N-hydroxy-PhIP by human hepatic microsomes and cytosolic O-acetyltransferase were higher than that for IQ and MeIQx and their N-hydroxy derivatives; with rat hepatic cytosols, the activation rate of N-hydroxy-PhIP was comparable to N-hydroxy-IQ, while it seemed to be lower than that of N-hydroxy-MeIQx (described in this study and in Ref. 9), suggesting that their organ-specific response is not likely to be due to their differential metabolic activation in the liver. However, our findings indicate that a disparity in the detoxification pathway for PhIP, IQ, and MeIQx may explain this phenomenon. In the liver, PhIP, IQ, and MeIQx are activated in a similar manner by N-hydroxylation and subsequent O-acetylation to form ultimate carcinogens, i.e., the N-acetoxy derivatives. Both N-acetoxy-IQ and N-acetoxy-MeIQx can then bind to DNA to form DNA adducts and presumably initiate hepatic tumorigenesis, whereas N-acetoxy-PhIP is converted to PhIP by the GST-catalyzed reaction with GSH. The PhIP formed from this reaction either is then detoxified by other inactivating pathways (e.g., 4'-hydroxylation and conjugation) or it reenters the activation/GST-dependent inactivation cycle by which it is finally removed from the liver.

Loretz and Pariza (41) observed that, in primary monolayer cultures of adult rat hepatocytes, depletion of cellular GSH by l-buthionine sulfoximine increased IQ binding to cell macromolecules; and addition of 10 mM GSH to the medium resulted in a slight decrease in the binding. Wallin et al. (34) also reported that a GST conjugate of MeIQx was formed in isolated rat hepatocytes. In our present study, GSH itself was indeed shown to partially inhibit DNA binding of activated IQ and MeIQx as well as PhIP (Tables 1 and 2; Fig. 2), suggesting that the electrophilic forms of all 3 heterocyclic amines are susceptible to nucleophilic attack by GSH. But it was found that only the reaction between activated PhIP and GSH can be catalyzed by GSTs. This different catalytic ability of the GSTs may reflect the substrate specificity and stability of the substrates as well, since it appears that N-acetoxy-PhIP is much more stable than N-acetoxy-IQ and N-acetoxy-MeIQx (cf. "Materials and Methods").

The mechanism of inactivation of N-acetoxy-PhIP by GSTs is not yet elucidated. HPLC analysis of the reaction mixture showed that the final product was PhIP. However, this does not exclude the formation of a labile conjugate. Stoichiometry of GSSG and PhIP formation (Table 3) suggested that the reaction was not a simple redox reaction. It has long been known that the α-class human GSTs are associated with Se-independent GSH peroxidase activity. However, using the GSH peroxidase from human erythrocytes (EC 1.1.1.9; Sigma), we did not find any activity of this enzyme toward N-acetoxy-PhIP, suggesting that peroxide reduction may not be involved in the catalytic mechanism of the reaction between N-acetoxy-PhIP and GSH. Also, we could find no evidence that N-acetoxy-PhIP is a substrate for Se-dependent GSH peroxidase in mouse liver cytosols under Se-deficient or Se-supplemented dietary conditions. Further experiments to understand this catalytic mechanism are needed.

Numerous studies on the tissue distribution of GSTs have found that striking differences in isozyme expression occur from one tissue to another (reviewed in Refs. 37 and 52); and GSTs of the α-class show the most restricted distribution. Immunochemistry of human α-GSTs have shown high levels only in liver (hepatocytes), kidney (proximal tubules), stomach, duodenum (epithelium), adrenal cortex (zona reticularis), testis ( Leydig cells), and pancreas (acinai cells). "Medium" levels are present in small intestine (epithelium) and

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Table 3  Formation of PhIP and GSSG in the GST-catalyzed reaction of N-acetoxy-PhIP with GSH

<table>
<thead>
<tr>
<th>GST 1-2 (μg/ml)</th>
<th>GSSG (μM)</th>
<th>PhIP (μM)</th>
<th>GSSG/PhIP</th>
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<tr>
<td>0</td>
<td>16.3 ± 8.0</td>
<td>32.1</td>
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<tr>
<td>3</td>
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<td>39.2</td>
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<td>9</td>
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<tr>
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<tr>
<td>60</td>
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<tr>
<td>120</td>
<td>39.5 ± 0.6</td>
<td>81.9</td>
<td>0.482</td>
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</table>

* Reaction mixtures, consisting of 100 mM phosphate buffer (pH 7.5), 100 μM [3H]-N-acetoxy-PhIP, 5 mM GSH, and 0.1 mM EDTA, and various amounts of rat GST 1-2, were incubated at 37°C for 30 min.

* Values are mean ± SD from 3 experiments.
thymus (57). Moreover, in the gastrointestinal tract, α-GSTs (including A1-1) decrease at least 100-fold from the duodenum to the colon (58–61); the mean liver content of GST subunit A1 is approximately 100-fold greater than in the colon (62).

These reports are clearly consistent with our observation of GST activity towards N-acetoxy-PhIP in liver but not in colon cytosols (Figs. 3 and 4). The distribution and diversity of the GST in any one tissue may be one of the critical determinants of its susceptibility to carcinogenesis. The results of previous studies in our laboratory on the distribution and diversity of the GST in any one tissue may be one of the critical determinants of its susceptibility to carcinogenesis (58—61); the mean liver content of GST subunit A1 is approximately 100-fold greater than in the colon (62).

Figs. 3 and 4). The distribution and diversity of the GST in any one tissue may be one of the critical determinants of its susceptibility to carcinogenesis. The results of previous studies in our laboratory on the distribution and diversity of the GST subunit A1 is approximately 100-fold greater than in the colon (62).

### GSTs and Human Carcinogenesis

GSTs can be induced by a number of chemicals including naturally occurring components of the human diet (63, 64). Dithiolthiones, among other compounds, were shown to be a potent inducer of mammalian and human α-class and other GSTs as well (47, 65), and this biochemical effect was correlated with their anticarcinogenic action (66). Cruciferous and yellow-green vegetables may contain dithiolthiones and other GST inducers, and epidemiological evidence has consistently indicated that regular consumption of these vegetables reduces the incidence of human colorectal cancer (reviewed in Ref. 67). In view of the role of GSTs in PhIP detoxification, specific vegetable consumption may provide effective protection against PhIP carcinogenesis by induction of GSTs.

In summary, the present study demonstrates the protection by human and rat α-class GSTs against the DNA damage induced in vitro by the ultimate carcinogen derived from the metabolism of PhIP but not from IQ and MeIQx. This detoxification pathway for PhIP may be an important factor that prevents its genotoxicity and carcinogenicity in the liver, where both the GST and GSH are abundant. However, in the colon, which is the carcino-gen target for PhIP, this protective factor is lacking. Based on our results, changes of organ specificity in the genotoxicity/carcinogenicity of PhIP by alterations of both GSH and α-class GST levels in vivo are expected. Studies are underway to examine this hypothesis.

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