Protection against 2-Hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine Cytotoxicity and DNA Adduct Formation in Human Prostate by Glutathione S-Transferase P1


ABSTRACT

The prostate has been identified as a target for 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced carcinogenesis. Humans are exposed to PhIP through ingestion of well-done cooked meats, and there is evidence from epidemiological studies that implicates red meat consumption in prostate carcinogenesis. The α and χ class isomers of glutathione S-transferases (GSTs) have been shown to inhibit adduction of activated PhIP metabolites to DNA in cell-free systems. In humans, silencing of GST χ (GSTP1) through CpG island hypermethylation is found in nearly all prostate carcinomas and is believed to be an early event in prostate carcinogenesis. We hypothesized that suppressed GSTP1 expression in prostate cells would increase their vulnerability to cytotoxicity and DNA adduct formation mediated by activated PhIP metabolites. To test this hypothesis, the human prostate adenocarcinoma cell line, LNCaP, which contains a silenced GSTP1 gene, was genetically modified to constitutively express high levels of GSTP1. Both LNCaP and LNCaP-GSTP1 cells exposed to N-OH-PhIP, but not parent PhIP, for 24 h showed a dose-dependent decrease in cell viability. GSTP1-overexpressing cells had LC50 s 30–40% higher than cells transfected with the vector alone. PhIP-DNA adducts isolated from LNCaP-derived cells and primary human prostate tissue cultures exposed to N-OH-PhIP were analyzed by liquid chromatography/electrospray ionization mass spectrometry. Primary cultures of human prostate tissue and LNCaP-GSTP1 cells had ~50% lower adduct levels than parental LNCaP and vector control cells. Bioactivation assays using LNCaP cytosols showed that enzymatic activation of N-OH-PhIP to a DNA binding species was dependent on ATP and could be inhibited by recombinant human GSTP1 in the presence of glutathione. This evidence confirms that N-OH-PhIP can be bioactivated to a DNA binding species in human prostate and human prostate-derived cells. These observations provide the basis for using LNCaP and LNCaP-GSTP1 cells as a model system for studying the role of this enzyme in protection against N-OH-PhIP induced DNA damage in prostate carcinogenesis. Loss of GSTP1 expression in human prostate may, therefore, enhance its susceptibility to carcinogenic insult by compounds such as N-OH-PhIP. Conversely, induction of GSTs in early-stage prostate carcinogenesis may be a useful protective strategy.

INTRODUCTION

Prostate cancer is the most frequently diagnosed lethal cancer in North American men, the second leading cause of cancer death, and has become a major health issue for men in the Western world. In the United States, >180,000 new cases and >31,000 deaths occur annually (1). The etiology of prostate cancer is still largely unknown; however, epidemiological evidence shows strong associations with dietary fat intake and red meat consumption (2). Although fat is believed to be the major component in red meat responsible for its observed association with prostate cancer, other compounds present in meats may also contribute to prostate carcinogenesis.

Heterocyclic amines are pyrosysis products that have been isolated from cooked fish and meat and have been shown to be highly mutagenic in the Ames assay and are carcinogenic in rodents. PhIP, the most abundant of the mutagenic heterocyclic amines found in cooked meats, has been shown to have the highest carcinogenic potential of these compounds to humans and has also been detected in cigarette smoke condensate (3, 4). Initial long-term dietary studies involving PhIP revealed it to be carcinogenic to the colon, mammary gland, and lymphoid system in rodents (5, 6). Evaluation of rat prostate tissues from the original mammary and colon carcinogenesis study has also implicated PhIP in the development of prostate cancer (7). Moreover, feeding of PhIP to lacI transgenic mice indicates that PhIP is a powerful prostate mutagen (8). The collective findings of high incidences of lesions and carcinomas of the ventral prostate along with high levels of PhIP-DNA adduct formation and mutagenesis in the rat prostate suggest that PhIP could be an important factor in the development of human prostate cancer.

Bioactivation of PhIP to a DNA binding species in target tissues is believed to play a major role in its carcinogenicity. N-Hydroxylation of PhIP by cytochrome P-450s (primarily CYP1A2) in the liver is an obligatory step in the pathway leading to DNA adduct formation. Subsequent esterification by NATs, STs, or kinases has been shown to lead to the formation of a major adduct at the C8 of 2’dG (reviewed in Ref. 9). Cell-free studies have shown that GSTs can inhibit the binding of N-acetoxy-PhIP to calf thymus DNA by as much as 90% (10). GSTP1, encoding the major GST isoform expressed in normal human prostate, is silenced by CpG island hypermethylation in nearly all prostate tumors and may be an early target for somatic alteration in multistep prostate carcinogenesis (11). This study sought to assess the impact of GSTP1 in prostate-derived cells on protection against cytotoxicity and DNA adduct formation by the prostate carcinogen, PhIP.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): calf thymus DNA, 2’dG, acetic anhydride, BSA, MTT, CDNB, ECA, GSH, AcCoA, PAPS, ATP, and DMSO. N-hydroxy-PhIP was obtained from the National Cancer Institute Chemical Carcinogen Reference Repository (Kansas City, MO). Nucleoside P1 was obtained from ICN Pharma-

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3 The abbreviations used are: PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; NAT, N-acetyltransferase; ST, sulfotransferase; 2’dG, 2’-deoxyguanosine; GST, glutathione S-transferase; N-OH-PhIP, 2-hydroxyamino-PhIP; MTT, 3-(4,5-dimethylthia-
zel-2-y1)-2,5-diphenyl tetrazolium bromide; CDNB, 1-chloro-2,4-dinitrobenzene; ECA, ethylenetricarbonyl; GSH, reduced glutathione; AcCoA, acetyl CoA; PAPS, 3’-phosphoadenosine 5’-phosphosulfate; rhGSTP1, recombinant human GSTP1; LC/ESI-MS, liquid chromatography/electrospray ionization mass spectrometry; 2’dG-C8-PhIP, N-(deoxyguanosino-8-yl)-PhIP.
were cultured (37°C, 5% CO2) in RPMI 1640 supplemented with 10% fetal
obtained from the American Type Culture Collection (Rockville, MD). Cells of PhIP or
described elsewhere, 4 were cultured in LNCaP medium containing 200
bovine serum (Life Technologies, Gaithersburg, MD), 100 units/ml penicillin,
bated for 1 h (37°C, 5% CO2) before the addition of fresh medium and

were cultured (37°C, 5% CO2) in RPMI 1640 supplemented with 10% fetal
obtained from men (age range, 51–67 years) treated for prostatic carcinoma by
bipolar prostatectomy at The Johns Hopkins Hospital (Baltimore, MD). Human
tissues, opposite neoplastic prostate, were dissected from resection specimens

tissue stimulation with a method described previously (14). Briefly, 100 nmol
N9-Oh-PhIP or DMSO:ethanol (4:1) vehicle in fresh medium for

were thawed and sonicated. The resultant homogenates were placed in Eppe-
dorf tubes and centrifuged at 10,000 × g. In the case of human prostate
cells, cytosols were prepared from fresh tissue, as described previously (10).
The supernatants were used for enzyme activity and protein determina-
tions. GST activity, expressed as pmol/min/mg cytosolic protein, was mea-
sured using CDNB and ECA as substrates to determine overall GST activity
and GSTP1-specific activity, respectively (12). Because of limited amounts of
human prostate tissues available and the known heterogeneity in GST isozyme
activity. Protein concentrations were determined using BCA Protein Assay
Reagent (Pierce, Rockford, IL) with BSA as the standard.

Western Blot Analysis. Cytosolic protein (50 μg), isolated and determined
as above for enzyme assays, was boiled for 15 min and resolved on a 12% SDS
polyacrylamide gel. After blotting, the membrane was blocked with 5% nonfat
dry milk in Tris-buffered saline containing 0.1% Tween 20, followed by a 1-h
incubation with rabbit anti-human GSTP1 serum (1:1000; Calbiochem, San
Diego, CA). The secondary horseradish peroxidase-labeled goat antirabbit IgG
(1:1000) was added for 1 h. The GSTP1 protein was visualized using the
supersignal chemiluminescent substrate of Pierce (Rockford, IL). Recombinant
human GSTP1 (Calbiochem, San Diego, CA) was boiled and loaded as a
positive control and comigrated with GSTP1 protein isolated from LNCaP
cells stably transfected with the GSTP1-expressing vector.

Cytotoxicity Assay. The colorimetric MTT assay measures the activity of
various dehydrogenase enzymes in active mitochondria in living cells and has
a linear relationship with cell number (13). Approximately 1 × 104 cells/well
were seeded in 96-well plates and treated 72 h later with graded concentrations of PhIP or N-OH-PhIP using DMSO:ethanol (4:1) as the vehicle at a final
concentration of 0.1%. After 24 h, the carcinoma was removed, and the plates
of cells were washed with PBS. Medium was added back to the plates, and 50
μl of MTT (2 mg/ml) in PBS were then incubated with the cells for 4 h at
37°C. The MTT and medium were removed, and DMSO was added to
solubilize the cells. The absorbance of the formazan product was detected
using a UV max Kinetic Microplate Reader (Molecular Devices, Menlo Park,
CA) set at a wavelength of 505 nm.

Synthesis of Adduct Standards. The 2’-dG-C8 adduct of PhIP was syn-
thesized by modifying a method described previously (14). Briefly, 100 nmol
of N-OH-PhIP in DMSO:ethanol (4:1) was reacted with 1 μl of acetic anhy-
dride on an ice-salt bath for 15 min under argon, resulting in the formation of
N-acetoxy-PhIP. The reaction mixture was then added to a 0.9 ml PBS solution
containing 2.4 mg of 2’dG and 0.4 mg of BSA (added to enhance the stability of
N-acetoxy-PhIP in solution, thereby increasing the yield of the end product)
and incubated at 37°C for 30 min in a shaking Eppendorf warmer. The protein
was then precipitated out using 2 volumes of ethanol and centrifugation at
12,000 × g for 10 min. The synthesized 2’-dG-C8-PhIP adduct was passed
through a Sep pack and purified by high-performance liquid chromatography
as described previously (14). The [15N2]-adduct was synthesized using the afore-
mentioned methodology; however [15N2]-2’dG was substituted for 2’dG.

DNA Addition and Isolation. LNCaP and LNCaP-GSTP1 cells (1 × 106)
were plated in 75-cm2 flasks and grown to ~75% confluence. Cells were then
treated with 20 μM N-OH-PhIP or DMSO:ethanol (4:1) vehicle in fresh medium
for 3 h. After the 3-h exposure, the cells were washed with ice-cold PBS, trypsinized,
harvested, centrifuged, washed again with ice-cold PBS, and then frozen in liquid
nrogen and stored at ~80°C until DNA isolation. Human prostate used for

N-OH-PhIP incubations were prepared and cultured as described above. After
the 3-h N-OH-PhIP exposure, the tissues were centrifuged, washed twice with ice-cold
PBS, frozen in liquid nitrogen, and stored at ~80°C until DNA isolation. Human prostate

tissues, or cells were thawed and homogenized in an ice-cold Teflon glass
homogenizer, and DNA was isolated using an A.S.A.P. Genomic DNA Isolation
kit (Boehringer Mannheim, Indianapolis, IN). DNA samples were washed twice
with 70% ethanol, resuspended in 30 μl sodium acetate/1 μl zinc sulfate buffer
(pH 5.3), and quantitated by measuring the absorbance at 260 nm, assuming 1
abundance unit = 50 μg/ml double-stranded DNA (A260/A280 ratios, 1.7–1.9).
DNA (~100 μg) from each sample was then digested with 10 units of nuclease P1
at 70°C for 2 h, followed by 10 units of alkaline phosphate at 70°C for 2 h.
Complete digestion to the resulting nucleoside bases was determined by high-
performance liquid chromatography with absorbance monitoring at 260 nm. The
ent standard (500 pg in methanol) was added to each sample after the
addition of 2 volumes of ethanol and centrifugation to precipitate out the protein.
The supernatants were evaporated to dryness in a rotary evaporator and
redissolved in 50 μl of methanol prior to LC/ESI-MS analysis.

LC/ESI-MS Analysis. Mass spectral data were acquired on an LCQ (Therm-
omet Corp., San Jose, CA) quadrupole ion trap mass analyzer equipped with an electrospray probe coupled to a Thermal Systems Products liquid
chromatography system, which consisted of a quaternary pump, an autosam-
pler, and a variable wavelength UV detector. An ODS J-sphere M-80 column
(2 × 250 mm; YMC Inc., Wilmington, NC) heated to 45°C was used for
sample separation. The mobile phase was as follows: A, mass spectrometry
grade H2O containing 0.1% formic acid; B, mass spectrometry grade methanol
containing 0.1% formic acid. The LC conditions were as follows: flow rate, 0.2
ml/min; a linear gradient from 0% B to 100% B in 10 min, isocratic B to 13
min, and linear to 0% B by 13.5 min to reset at starting conditions. Collision-
induced dissociation and selected reaction monitoring were optimized for analysis
of the 2’dG-C8-PhIP adduct, and a standard curve was generated based on
the signal area of known concentrations. Recovery of the [15N2]-
internal standard was also monitored in the same chromatographic run for each
sample and was highly variable as a result of sample preparation and recon-
stitution in methanol. 2’dG was analyzed by LC/ESI-MS under the same
chromatographic and mass spectral conditions. Data are represented as pmol
2’dG-C8-PhIP (corrected for recovery of internal standard) per μmol 2’dG,
with a detection limit of 0.7 pmol adduct per μmol 2’dG (equivalent to ~2
adducts/107 nucleotides).

Measurement of Cellular AcCoA and ATP in LNCap Cells. LNCap
cells in log growth were assayed for AcCoA by reversed-phase high-perfor-
mance liquid chromatography as described previously (15). LNCap cells at
1.5 × 105 cells/ml were plated into 96-well plates with each well containing
0.2 ml of cell suspension. Forty-eight hours later, cellular ATP content was
determined using a luciferin-luciferase ATP assay kit (bioluminescent somatic
cell assay kit; Sigma) as described previously (16).

Cytotoxic Activation and Inhibition Assays. Assays were conducted to
determine relative contributions of NAT, ST, and ATP-dependent transfer(s) in
the bioactivation of N-OH-PhIP to a DNA binding species by LNCap cytosol
using enzyme-specific cofactors as described previously, with modifications
(17), and to assess the ability of rhGSTP1 (Calbiochem, San Diego, CA), allele
unknown, to inhibit bioactivation. Covalent binding of N-OH-PhIP (20

4 Manuscript in preparation.
GST Activity and Protein Expression in LNCaP and LNCaP-GSTP1 Cells. Single clones of cells transfected to stably express GSTP1 (GSTP1-C1 and GSTP1-C5) were assessed for GST activity and compared with parental LNCaP cells and vector control cells, LNCaP-Neo. Cytosols isolated from LNCaP and LNCaP-Neo cells had very low basal levels of GST activity, whereas GSTP1-C1 and GSTP1-C5 both showed significantly higher overall conjugating activity with CDNB as substrate, 6.1- and 20.2-fold, respectively, compared with LNCaP-Neo (Fig. 1A). GSTP1-specific activity, determined using ECA as the substrate, was significantly higher in both GSTP1-C1 and GSTP1-C5 cytosols, 7.5- and 20.5-fold, respectively, compared with LNCaP-Neo (Fig. 1B). Assessment of GSTP1 content in LNCaP, LNCaP-Neo, GSTP1-C1, and GSTP1-C5 cells by Western blot analysis indicates that LNCaP and LNCaP-Neo cells have essentially no expression of GSTP1 protein, whereas GSTP1-C1 and GSTP1-C5 cells have progressively higher levels of protein expression that coincide with the activity assays (Fig. 1C). Western blot analysis for α class GST was weakly positive in LNCaP cells (data not shown) and may account for the basal level of GST activity in these cells.

GST Activity and Detection in Human Prostate. Overall GST activity, using CDNB as the substrate, in human prostate cytosols showed a range of values from 61 to 227 with a mean of 162 nmol/min/mg protein and were consistent with literature values (18). This level of overall GST activity is comparable with the levels seen in the GSTP1-overexpressing cell line GSTP1-C1 (mean, 264 nmol/min/mg protein). However, immunohistochemical analysis of the human prostate samples indicated that at least three different isoforms (α, μ, and π) of GST were expressed at varied levels in the epithelial cells of the peripheral zone (data not shown).

Cytotoxicity of N-OH-PhIP and PhIP in Parental LNCaP Cells and LNCaP Cells with Elevated GSTP1 Expression. The MTT assay revealed that exposure to N-OH-PhIP (0–50 μM) for 24 h was cytotoxic to all four cell lines in a concentration-dependent manner (Fig. 4). Both clones of LNCaP-GSTP1 cells were more resistant to cytotoxicity compared with LNCaP or LNCaP-Neo cells. The concentration of N-OH-PhIP required to kill 50% of the cells (LC50) increased significantly by 34 and 40% in GSTP1-C1 and GSTP1-C5 cells, respectively, compared with LNCaP-Neo cells (P < 0.05, paired Student’s t test; Fig. 2, inset). Exposure of LNCaP cells to PhIP for 24 h at concentrations as high as 100 μM showed no concentration-dependent cytotoxicity (Fig. 2).

DNA Adduct Analysis of LNCaP-derived Cells and Human Prostate Exposed to N-OH-PhIP in Culture. DNA samples isolated from human prostate primary cultures and LNCaP-derived cells exposed to 20 μM N-OH-PhIP were analyzed for the presence of 2′dG-C8-PhIP by LC/ESI-MS. This 2′dG-C8-PhIP adduct has been characterized previously by LC/ESI-MS (19) and is the predominant adduct seen in in vitro and in vivo systems. Under the LC/MS conditions described in “Materials and Methods,” digested DNA from exposed tissues and cells contained a major peak that coeluted with synthesized 2′dG-C8-PhIP and [15N5]-adduct standards (Fig. 3). Although the LC/ESI-MS method developed was highly specific for the adducts measured, some minor peaks that did not correspond to the elution of known standards were observed in chromatographic runs. These minor peaks were also observed in some blank runs and in digested DNA samples from LNCaP cells exposed to vehicle alone. 2′dG-C8-PhIP formation, expressed as pmol per normal 2′dG (μmol) and corrected for the percentage of recovery of the internal standard, in LNCaP cells was concentration dependent over the range of 3–100 μM (data not shown). Shown in Fig. 4, transfected cells that overexpressed GSTP1 had adduct levels nearly one-half the amount seen in parental LNCaP and LNCaP-Neo cells. 2′dG-C8-PhIP adduct levels in primary cultures of human prostate were on average 50% lower than levels seen in LNCaP or LNCaP-Neo cells. The human prostate tissues used for GST activity measurements and N-OH-PhIP exposure in culture were the same. No correlation between levels of 2′dG-C8-PhIP formation and GST activity or expression was observed. Human prostate tissues not exposed to N-OH-PhIP in culture had no detect-
able background levels of 2′dG-C8-PhIP. Moreover, treatment of LNCaP cells or primary human prostate tissues with 100 μM PhIP for up to 6 h also did not yield any detectable 2′dG-C8-PhIP.

Bioactivation of N-OH-PhIP by LNCaP Cytosol and Inhibition by GSTP1. Assays were performed with cytosolic fractions of LNCaP cells to determine the possible pathways involved in bioactivation of N-OH-PhIP to a DNA binding species (Fig. 5A). Of the three major cofactors (PAPS, ATP, and AccoA) used in these determinations, the only one that enhanced binding to calf thymus DNA, as determined by formation of 2′dG-C8-PhIP, compared with controls was ATP. Slightly higher binding, compared with control incubations with no cofactor, was seen in the presence of AccoA, but these levels were also seen in the heat-inactivated cytosol control. The physiological levels of AccoA and ATP measured in LNCaP cells were 6.1 ± 0.5 μM (mean ± SE; n = 3) and 1.8 ± 0.1 mM (mean ± SE; n = 5), respectively. Assay conditions that involved concentrations of AccoA >100-fold over observed physiological levels did not significantly elevate the formation of 2′dG-C8-PhIP, whereas concentrations of ATP that were actually less than observed physiological levels produced significant adduct formation with native LNCaP cytosol. ATP-dependent 2′dG-C8-PhIP formation was significantly inhibited by GSH alone, but this inhibition was extended to near background levels by the addition of rhGSTP1 (Fig. 5B).

**DISCUSSION**

The prostate has been identified as a target for PhIP-induced carcinogenesis, DNA adduct formation, and mutagenesis in rodents (7, 8). The positive associations with prostate cancers and red meat consumption seen in epidemiological studies (2) support the assumption that heterocyclic amines, such as PhIP, may play a role in the etiology of human prostate cancer. Evidence presented here indicates that human prostate and human prostate-derived cells have the capacity to bioactivate N-OH-PhIP, but not PhIP, to cytotoxic and DNA-damaging species(s). Kaderlik et al. (20) reported that i.v. administration of N-OH-PhIP to rats led to high levels of PhIP-DNA adducts in all of the extrahepatic tissues examined. This evidence indicates that N-OH-PhIP, believed to be formed primarily in the liver, is sufficiently stable to be transported through the circulation and further metabolized to a DNA-binding species in distal organs such as the prostate.

We have developed a very sensitive and highly specific LC/ESEI-MS methodology for detecting and quantitating the major PhIP-DNA adduct, 2′dG-C8-PhIP, formed in *in vivo* and *in vitro* systems. No other DNA adducts were detected by LC/ESEI-MS; however, it should be noted that the conditions were optimized for detecting the 2′dG-C8-PhIP adduct. This method was used to measure PhIP-DNA adduct formation in prostate-derived cells and in a cell-free system through bioactivation of N-OH-PhIP. Our data (Fig. 5A) suggest that the pathway leading to DNA adduct formation in LNCaP cells involves ATP-dependent enzymes. One ATP-dependent enzyme believed to be involved in N-OH-PhIP activation is tRNA synthetase/kinase, but the evidence is inconclusive as to whether activation is attributable solely to the tRNA synthetase/kinase or to other kinases as well (17, 21). The highly reactive derivatives resulting from this ATP-dependent activation of N-OH-PhIP are believed to be either the N-prolyloxy or the N-phosphatyl esters at the exocyclic amino group. These ester moieties serve as leaving groups, giving rise to putative electrophilic arylnitrenium ion intermediates, which subsequently bind to the C8 position of guanine (9). This ATP-dependent activation of N-OH-PhIP has been observed in various tissues of humans, rodents, and nonhuman primates (17, 21, 22) and may play a significant role in the susceptibility of extrahepatic tissues to heterocyclic amine-induced DNA adduct formation, particularly in tissues where NAT and ST activities are low. Activation of heterocyclic amines by NATs and STs has been demonstrated in cytosolic fractions isolated from human colon and mammary cells (21, 23–25). NAT-dependent O-acetylation of heterocyclic amines is believed to be an important factor in the susceptibility of the colon and mammary gland to heterocyclic amine-induced carcinogenesis (21, 23, 25). There is recent evidence showing the presence of NAT mRNA in human prostate epithelium (26), which may account for some activation of N-OH-PhIP *in situ*. However, Leff et al. (27) have shown that overexpression of human NAT2, the isofrom with the highest relative capacity to convert N-OH-PhIP to a DNA-binding species (28), specifically in the prostate of transgenic mice, does not enhance PhIP-DNA adduct formation in that tissue. This evidence, along with our findings, suggests that this ATP-dependent activation pathway may be very important in determining prostate-related toxicities in contrast to colonic and mammary tissues.

LNCaP cells, a well-characterized prostate adenocarcinoma cell line, serve as a model for understanding many aspects of prostatic epithelial cell biochemistry and molecular biology. These cells have an experimentally useful feature in that they possess a GSTP1 gene that has been silenced because of CpG island hypermethylation of the promoter region. This silencing, which occurs in >90% of prostatic carcinomas and is increased in prostatic intraepithelial neoplasia, may be an early genetic lesion that predisposes select cells to carcinogenic insult and may be useful as a biomarker of early-stage prostate carcinoma (11, 29). Restored expression of GSTP1 in LNCaP cells has allowed us to explore the ability of GSTP1 to protect these prostate-derived cells from toxicities associated with exposure to a potential human prostate carcinogen. Our results show that overexpression of GSTP1 in LNCaP cells does partially protect against cytotoxicity and DNA adduct formation caused by exposure to N-OH-PhIP. The observed cytotoxicity suggests, but does not definitively
prove, that these prostate-derived cells express enzymes that can bioactivate N-OH-PhIP to an esterified form, which could in turn bind to cellular macromolecules and cause cell death. The relevance of this model system is further supported by the observation that 2'dG-C8-PhIP levels seen in human prostate surgical samples exposed to N-OH-PhIP at equivalent concentrations and time points are comparable with levels seen in GSTP1-overexpressing cells (Fig. 4). This feature not withstanding, there remain obvious difficulties in comparing data from prostate cancer cells grown in culture to prostate tissue that contains a heterogeneous population of cells. Differences in activation enzymes as well as detoxification enzymes (e.g., the varied expression of GST isoforms) between LNCaP cells and human prostate tissue could potentially affect the cytotoxicity and adduct formation caused by N-OH-PhIP. In fact, no correlation between levels of 2'dG-C8-PhIP formation and GST activity or GST expression in human prostate cultures was observed. Because of the heterogeneity of GST isozyme expression in any given human prostate tissue section, along with the possibility for differences in N-OH-PhIP activation, associations between DNA adduct formation and qualitative enzyme expression in individual tissues are difficult. Immunohistochemical staining of GSTP1-C1-overexpressing cells showed that there is very heterogeneous expression of the protein, with some cells expressing very high levels whereas others express either very low or intermediate levels, somewhat mimicking the in vivo situation. Although derived from clonal expansion of a single colony, heterogeneous expression of GSTP1 may be caused by differential regulation in individual cells.

\footnote{Unpublished data.}
A previous study has shown that several GST isoforms were able to inhibit DNA binding of N-acetoxy-PhIP (an ultimate DNA binding species) to calf thymus DNA by as much as 90% (10). Specifically, GSTP1–1 was able to significantly inhibit binding by 30%. Our results confirm that GSTP1 can inhibit binding of activated PhIP metabolites to DNA in cellular and cell-free systems and that there may be biological relevance in the prevention of toxicities to the prostate. GSH, a highly abundant cellular nucleophile, alone was able to significantly inhibit ATP-dependent adduct formation in a cell-free system (Fig. 5B). This result could be attributable to nonenzymatic binding of GSH to the reactive intermediate(s) or to low levels of other GST isoforms (low levels of α class GST were detected by Western blot analysis) present in LNCaP cytosol binding to and inactivating the reactive intermediate(s). The mechanism of inactivation of N-esterified-PhIP intermediates has not yet been determined, but Lin et al. (10) demonstrated that the end products of a reaction mixture that contained N-acetoxy-PhIP, GST, and GSH were PhIP and oxidized glutathione. The stoichiometry of the end products, PhIP and oxidized glutathione, suggested that the reaction was not a simple redox reaction. There is evidence that a glutathione conjugate is formed, but that it is highly labile and degrades to form a 5-hydroxy-PhIP metabolite (30). Our observations of nearly complete inhibition of ATP-dependent N-OH-PhIP-induced DNA adduct formation in a cell-free system by GSTP1 suggests that this GST isoform may have a higher substrate specificity for the ATP-dependent metabolite(s) of N-OH-PhIP versus N-acetoxy-PhIP, thus enhancing its ability to inhibit binding to DNA. Further studies should focus on determining the enzyme(s) responsible for producing the ultimate DNA binding species in prostate-derived cells as well as characterizing the structural properties of the activated metabolite(s). The effectiveness of other GST isoforms should also be assessed in their ability to inhibit the cytotoxic and genotoxic effects of activated PhIP metabolites in the prostate.

GSTs have been proposed to play a critical role in defending normal cells against electrophilic carcinogens. Inactivation of GSTP1 in prostate cells by promoter hypermethylation may lead to increased vulnerability to electrophilic carcinogens. We have demonstrated that restored GSTP1 expression in the prostate-derived cell line LNCaP can inhibit cytotoxicity and DNA adduct formation caused by a potential dietary carcinogen. The evidence presented leads to the possibility of strategies for the prevention of initial and cumulative DNA-damaging events caused by N-OH-PhIP and other similar compounds that may lead to multistage prostate carcinogenesis. A recent study by Montironi et al. (31) suggests that finasteride, a 5α-reductase inhibitor currently used in a human prostate cancer prevention trial, may act as a GST π inducer in human prostate. Induction of GSTs in the prostate by chemopreventive agents may therefore be a viable preventive strategy, either alone or in concert with other mechanisms. This line of reasoning may also help to explain the negative correlation of prostate cancer with high vegetable intake seen in epidemiological studies (2, 32), because these vegetables contain potent inducers of GSTs such as isothiocyanates (33).

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Protection against 2-Hydroxyamino-1-methyl-6-phenylimidazo[4,5- \textit{b}]pyridine Cytotoxicity and DNA Adduct Formation in Human Prostate by Glutathione S-Transferase P1

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