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Benzo[a]pyrene Induction of Glutathione S-transferases: An activity-based protein profiling investigation

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants generated from combustion of carbon-based matter. Upon ingestion these molecules can be bioactivated by cytochrome P450 monooxygenases to oxidized toxic metabolites. Some of these metabolites are potent carcinogens that can form irreversible adducts with DNA and other biological macromolecules. Conjugative enzymes, such as glutathione S-transferases or UDP-glucuronosyltransferases, are responsible for the detoxification and/or facilitate the elimination of these carcinogens. While responses to PAH exposures have been extensively studied for the bioactivating cytochrome P450 enzymes, much less is known regarding the response of glutathione S-transferases in mammalian systems. In this study, we investigated the expression and activity responses of murine hepatic glutathione S-transferases to benzo[a]pyrene exposure using global proteomics and activity-based protein profiling for chemoproteomics, respectively. Using this approach, we identified several enzymes exhibiting increased activity including GSTA2, M1, M2, M4, M6, and P1. The activity of one GST enzyme, GSTA4, was found to be downregulated with increasing B[a]P dose. Activity responses of several of these enzymes were identified as

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Author Contributions

The manuscript was written through contributions of all authors. JNS, ATW, EGS, SAN, RAC designed the experiments. EGS, JM, SAN conducted experiments. Probe synthesized by BJK. EGS, ATW analyzed proteomics and SDS-PAGE data. All authors have given approval to the final version of the manuscript.

The authors declare no competing financial interests. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE⁵⁹ partner repository with the dataset identifier PXD010610 and 10.6019/PXD010610.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Table S1. Results of linear regression analyses (.xlsx)

Figure S1. Coomassie total protein stain of GSTABP-G labeling of microsomes from B[a]P treated mice (.tif)

Figure S2. Coomassie total protein stain of GSTABP-H labeling of microsomes from B[a]P treated mice (.tif)

Figure S3. Curve fits from linear regression analyses of proteomics data (.pdf) Linear regression curves were fit plotting B[a]P dose (on log2 scaled x-axis) and relative abundance values (plotted on log10 scaled y-axis). Blue represents data from GSTABP-H enriched proteomics samples. Green represents data from GSTABP-G enriched proteomics samples. Red represents data from global proteomics samples.

being expression-independent when comparing global and activity-based datasets, possibly alluding to as of yet unknown regulatory post-translational mechanisms.

Graphical Abstract

Keywords

polycyclic aromatic hydrocarbons; benzo[a]pyrene; chemoproteomics; glutathione-S-transferase

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of ubiquitously found environmental contaminants generated by exposing carbon-based materials to very high heat or combustion.¹ Many common sources of PAH exposure come from anthropogenic processes such as combustion of fossil fuels, grilling or smoking meat, smoking, and other industrial processes.^{2–4} Upon ingestion, some relatively non-toxic PAHs are converted to their toxic metabolites by cytochrome P450s. These biotransformation enzymes oxygenate PAHs to a variety of hydroxylated metabolites, diols, epoxides, diol epoxides, and diones.^{5, 6} One product of the P450 monooxygenation of benzo[a]pyrene (B[a]P) is the prototypical PAH carcinogen, benzo[a]pyrene-7,8-dihydrodiol-9,10-oxide (BPDE). Not only are some PAHs bioactivated to highly carcinogenic metabolites by P450s, they also perpetuate further increases in their own bioactivation via P450 induction⁷. While P450 bioactivation and induction results in the production of carcinogenic metabolites, detoxifying enzymes convert these metabolites into non-toxic products and facilitate their expulsion. These enzymes include UDP glucuronosyl-transferases, sulfotransferases, and glutathione S-transferases.

Glutathione S-transferases conjugate glutathione to a diverse group of xenobiotics. The active site of GSTs consists of two distinct subsites: the glutathione-binding G site and substrate-binding H site.⁸ The primary role of GSTs in PAH metabolism is thought to be the glutathione conjugation of PAH metabolites, such as epoxides and diol epoxides.^{9–15} Induction of GST expression is thought to be caused by changes in redox conditions; for instance, one study showed that the Aryl hydrocarbon receptor (Ahr) mediated little induction of GSTs, and that induction via NFE2-related factor 2 (Nrf2) is much more potent.

¹⁶ Previously, GST induction by polycyclic aromatic hydrocarbons (PAHs) has been measured by determining mRNA levels, by western blotting for protein abundance, via GSH-agarose column enriched samples, and/or by GST activity via simple colorimetric assays.^{17–19} Western blotting and measurement of mRNA levels provide information regarding the expression but are not necessarily indicative of enzyme activities. Utilizing GSH-agarose columns to enrich induced samples provides meaningful insight into GSTs that maintain their GSH binding activity, but do not explore the activity of GST H-sites. The studies referenced above measure total GST activity via colorimetric assay and though some isoform and class-specific assays are available, many GST isoforms still lack specific assays in part due to high substrate overlap of different GSTs of the same class.^{20–23} In a broader sense, the majority of investigations into the role of GSTs following PAH exposure have been performed using mammalian cell lines.^{24–26} These models of induction do not take into account many complex factors in a mammalian system including absorption, distribution, and excretion, all of which modulate the internal dose observed by individual organs.

Activity-based protein profiling (ABPP) is a proven method to enrich and quantify enzymes based upon functional activity in biological systems.²⁷ The ABPP approach uses activity-based probes (ABPs) designed to target and covalently bind enzymes of a specific function. Recently, we reported on the synthesis and validation of two ABPs that irreversibly bind and target GSTs and report on their enzymatic activity in mammalian tissues.²⁸ One probe, **GSTABP-G**, features a glutathione molecule to which benzophenone and alkyne moieties have been appended to the functional thiol of glutathione. This ABP effectively investigates the glutathione binding activities of GSTs. The second probe, **GSTABP-H**, is based on the known suicide inhibitor of GSTs, 2,3-dichloro-1,4-naphthoquinone. This probe has been validated to bind active GSTs in substrate binding H sites. Both probes feature an alkyne handle to facilitate click chemistry²⁹ mediated addition of azide appended reporter groups such as TAMRA-azide (for visualization of probe targets by SDS-PAGE and fluorescent detection) or biotin-azide (for streptavidin-biotin enrichment). The use of these probes offers a unique look into the activity of these phase II biotransformation enzymes that is not possible using other methods to measure activity.

The purpose of this study was to investigate B[a]P induction of active hepatic GSTs using chemoproteomic probes. This activity-based proteomics approach results in investigation of activity with improved resolution that allows for a much closer investigation into susceptibilities associated with xenobiotic exposures. While methods such as measurement of GST mRNA levels and western blotting have provided information regarding induction of GST expression by PAHs, they provide no indication of enzyme-specific activity. In addition, mice were used as an *in vivo* animal model to capture full organismal response, which cannot be replicated in cell culture systems. Herein, using ABPP in tandem with global proteomics analyses we investigated B[a]P-specific GST induction of both active GST G and H sites in the liver.

Materials and Methods

Animals

Our group has utilized female B6129SF1/J mice in prior PAH carcinogenicity, toxicokinetic, and in vitro metabolism studies,^{30–34} and was subsequently chosen for this study. Adult female B6129SF1/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were fed Lab Certified rodent chow (PMI Nutrition International Certified Rodent Diet 5002) ad libitum.

Effects of PAHs on GST induction was evaluated using a serial sacrifice design. After acclimation for 1 week, mice were dosed with a water vehicle, a corn oil vehicle, or B[a]P in a corn oil vehicle by oral gavage at 2, 20, or 180 $\mu\text{mol/kg}$ every 24 hr for 3 days (at $t = 0$ hr, $t = 24$ hr, $t = 48$ hr). Four mice were dosed per dosing level. After 72 hr (24 hr after the third consecutive dose), mice were euthanized by CO_2 asphyxiation and cervical dislocation. After euthanization, livers were rapidly removed, rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.4), frozen in liquid nitrogen, and stored at -70°C . All protocols were approved by the Pacific Northwest National Laboratory Institutional Animal Care and Use Committee.

Preparation of liver cytosolic fraction

Hepatic microsomes were prepared by differential centrifugation. Livers ($n=4$) from each dosing condition were pooled and minced using a tissue tearor. Minced liver tissue was subsequently homogenized in sucrose (250mM) in PBS solution using a glass dounce homogenizer with 15 pulses each from loose and tight-fitting pestles. Whole liver lysate was centrifuged at $10,000 \times g$ for 25 min at 4°C . The supernatant S9 fraction was collected and centrifuged again at $100,000 \times g$ to separate the cytosolic (supernatant) and microsomal (pellet) fractions. Cytosolic proteins were quantified via the bicinchoninic acid assay (BCA).³⁵

Activity-based probe labeling and click chemistry

GST enzymes were labeled using two different ABPs. Cytosolic hepatic proteins were normalized to 1 mg/mL protein (2 mg/mL for SDS-PAGE analysis). 100 μg (for SDS-PAGE gels) or 500 μg (for streptavidin enrichment for MS-based chemoproteomics) were incubated with **GSTABP-G** (20 μM), **GSTABP-H** (20 μM), or DMSO only controls for 30 min at 37°C , shaking at 1000 rpm. **GSTABP-G** labeled proteins were then UV exposed for 7 min to activate the benzophenone for covalent labeling of protein targets. Following probe incubation, click chemistry reactions were conducted on all samples. The following reagents were added to each sample in the following order: (1) TAMRA-azide (60 μM) for SDS-PAGE analysis or biotin-azide (60 μM) for biotin streptavidin enrichment, sodium ascorbate (10 mM), tris-hydroxypropyltriazolylmethylamine (2 mM), and CuSO_4 (4 mM). Samples were incubated at room temperature for 90 min. Following click chemistry, ice cold MeOH (60 %) was added to each sample. Samples were then placed in a -70°C freezer for 1 hr to induce further protein precipitation. Precipitated proteins were pelleted via centrifugation at $14,000 \times g$ for 10 min and the supernatant was discarded. Proteins were resolubilized by adding SDS (1.2 %) in PBS, sonicated with 2×1 s pulses at 60 % amplitude. Samples were

then heated at 95 °C for 2 min. Protein from samples undergoing streptavidin enrichment were quantified via BCA.

SDS-PAGE

SDS-PAGE was used to visualize activity-based probe labeling. Probe labeled, TAMRA appended proteins were resolved via SDS-PAGE. 2X NuPage Running Buffer and 10X NuPage Reducing Agent were added to resolubilized protein samples. 20 µg protein was loaded into each well of 4 – 12 % Bis-Tris gel cassettes. Gels were run at 150 V, 35 mA for 1 hr in MES buffer. Gels were imaged using a Typhoon FLA 9500 (General Electric). Fluorescent bands were quantified using ImageJ software. After imaging, gels were fixed with a solution of MeOH (50 %), acetic acid (7 %), and water (43 %) and then stained with GelCode Blue. Stained gels were rinsed with water and imaged using GelDocEZ (BioRad Laboratories).

Enrichment of ABP Targets for MS Analysis

Probe labeled, biotin appended proteins were normalized to 450 µg protein per sample, in SDS (1.2 %) in PBS solution. 100 µL streptavidin agarose resin (binding capacity 1–3 mg protein/ 1 mL resin) was washed with SDS (0.5 %) in PBS, urea (6M) in NH₄HCO₃ (25 mM, pH = 8), and PBS. Beads were incubated with samples in PBS at 37 °C, with rotation, for 1 hr. Following enrichment, beads were transferred to fritted columns (BioRad) and washed under vacuum with SDS (0.5 %) in PBS, urea (6M) in NH₄HCO₃ (25 mM), PBS, NH₄HCO₃ (25mM) and MilliQ water. Beads were then suspended in urea (6M) in NH₄HCO₃ (25 mM). Beads were incubated with tris(2-carboxyethyl)phosphine hydrochloride (5mM) at 37 °C for 30 min to reduce the samples followed by incubation with iodoacetamide (10 mM) at 50 °C for 45 min to alkylate samples. Beads were again placed on the fritted columns and washed under vacuum with PBS and NH₄HCO₃ (25 mM). On-bead trypsin digestion was carried out overnight with 0.125 µg trypsin (Promega) in NH₄HCO₃ (25 mM, pH = 8.0) at 37 °C, with rotation. Trypsinized peptides were collected and dried using a speedvac concentrator. Dried peptides were resuspended in NH₄HCO₃ (25 mM) and analyzed via LC-MS/MS.

Preparation of peptides for global proteomics analysis

Cytosolic proteins from all samples were normalized to 1 mg/mL protein concentration. Urea (6 M) was added to 100 µL normalized protein. Protein was reduced with dithiothreitol (5 mM) at 60 °C for 30 min. Samples were subsequently alkylated with iodoacetamide (40 mM) for 1 hr at 37 °C. Samples were then diluted 10-fold with NH₄HCO₃ and incubated with 2 µg trypsin (Promega) for 3 hr at 37 °C. Digested peptides were washed on Discovery C18 SPE columns conditioned and equilibrated with methanol and trifluoroacetic acid (0.1%), respectively. After capture on the columns peptides were washed with acetonitrile (5 %) in water and eluted with acetonitrile (80 %) in water. Eluted peptides were dried using a speedvac concentrator, resuspended in NH₄HCO₃, and normalized to 0.1 mg/mL peptide concentration (via the BCA assay) for LC-MS/MS analysis.

Proteomics data analysis

All peptides were analyzed on a Velos Orbitrap mass spectrometer as outlined in Sadler et. al.³⁶ The resulting peptide spectra were searched against the Uniprot³⁷ *mus_musculus* database and rescored using MSGF +.³⁸ Following that, an AMT tag analysis was conducted as previously described.³⁹ Only unique peptides with MT FDR threshold 0.1 and MT Uniqueness 0.5 were used in further analysis. Peptides meeting these criteria were log₂ transformed and rolled up to the protein level using Inferno RRollup software.⁴⁰ Enrichment of GST targets were validated using paired student's t-test and fold change analysis of probe vs. no probe samples. Treatment effects were evaluated by testing the slope of a linear regression model and comparing fold changes across treatments. Fold changes >2 and *p*-values <0.05 were used as thresholds in determining significant dose dependence. Pearson correlation analysis was also conducted to investigate relationships of **GSTABP-G** and **GSTABP-H** biotin-streptavidin enriched samples and global proteomics samples.

Results and Discussion

B[a]P induction of GSTs measured via gel electrophoresis

We investigated total probe labeling of GSTs by GSTABP-G and H ABPs in vehicle and B[a]P treated samples. This was measured via fluorescence of probe labeled/TAMRA appended proteins resolved via SDS-PAGE (Figure 1, Figure S1, Figure S2). No apparent differences in probe-measured total GST activities were observed between water and corn oil vehicle. Though no obvious B[a]P dependent changes in probe-determined GST activity were observed, fluorescence was increased over corn oil vehicle dosed samples by 61% in GSTABP-H probed liver cytosol from mice treated with 180 µmol/kg.

While apparent increases in GST fluorescence observed by both probes were minimal, many GSTs contribute to the total fluorescence at 25 kDa and resolution of individual GSTs is not possible. Due to differences in affinity of individual GSTs for the probes, as well as large differences in expression between them, minimal changes seen via gel don't necessarily equate to minimal changes in the activities of individual GSTs. Similar method-inherent caveats apply when using commercially-available GST activity assays measuring DNCB or ethacrynic acid glutathione conjugation. Thus, to determine individual GSTs responsible for these observed changes in probe-mediated fluorescence, we enriched probe-targeted GSTs in these B[a]P treated samples.

ABPP of liver cytosolic GSTs

Hepatic cytosolic GSTs from water, corn oil, and B[a]P dosed mice (2, 20, 180 µmol/kg) were enriched in murine liver lysate by labeling with 20 µM of each GSTABP followed by the addition of a biotin molecule via click chemistry. Proteins were then incubated with streptavidin agarose beads and subsequently enriched for LC-MS/MS analysis. Both probes significantly enriched 14 GSTs as depicted in Table 1. These GSTs included GSTA2, A3, A4, K1, M1, M2, M4, M5, M6, M7, P1, T1, T2, and O1. Significant enrichment of these enzymes was determined via fold change analysis and paired student's t-tests comparing enriched versus corresponding no probe controls for all doses/vehicle controls. Enrichment of GST targets was highly significant with *p*-values for the majority of enzymes well below

0.001 and fold changes greater than 3 and as high as 1400. All 14 of these enzymes were also detected in global proteomic analysis of the same lysates. In total, the probe enrichment yielded excellent coverage of GSTs (14/19 of the total number of mammalian cytosolic GSTs) for an effective and broad analysis of the inductive capacity of B[a]P in murine liver.

B[a]P induced changes in activity of GST G sites

B[a]P induced active G sites of several enzymes as measured by **GSTABP-G**. Significantly induced enzymes include GSTA2, GSTM4, and GSTP1. Significance of induction was determined via linear regression analysis ($p < 0.05$) and fold change abundances of 180 $\mu\text{mol/kg}$ dose versus corn oil controls (fold change > 2). GSTA2 showed 2.7, 2.0, and 3.3-fold increases in 2, 20, and 180 $\mu\text{mol/kg}$ B[a]P treated samples, respectively. GSTP1 was induced 2.3- and 2.5-fold at 20 and 180 $\mu\text{mol/kg}$ B[a]P doses, respectively. Interestingly, GSTA4 showed significant downregulation as determined by fold change and linear regression analysis with 2.5-fold inhibition at 180 $\mu\text{mol/kg}$ B[a]P (Figure 2, Table S1, Figure S3).

The glutathione binding sites of cytosolic GSTs are composed of a set of highly conserved residues that non-covalently orient glutathione for optimal nucleophilic attack of electrophiles in adjacent substrate binding sites.⁸ Glutathione is a tripeptide consisting of glutamine, cysteine, and glycine. Concentration of glutathione in eukaryotic cells typically ranges from 5–10 mM.⁴¹ The sulfhydryl from the cysteine residue of glutathione acts as a nucleophile in the attack of electrophilic xeno- and endo-biotic substrates.⁸ Changes in enrichment of individual GSTs with B[a]P dose, as measured by the glutathione derived activity-based probe, is likely due to either increased expression or could be due to some modification following translation of the enzyme that increases GST affinity for GSH (thus increasing probe binding). Some post-translational modifications of GSTs are known and include the phosphorylation of serines, tyrosines, and threonines of GSTs.^{37, 42} Phosphorylation of some GSTP1 residues are known to increase metabolic activity.⁴³ Additionally, cysteine residues of GSTP1 are known to be glutathionylated.⁴⁴ Conversely, these modifications are thought to result in decreases in activity. Distinction between expression-derived G site induction or induction via modification of expressed enzyme can be ascertained by comparing enzyme expression versus probe-determined G site activity (described below).

B[a]P induced changes in activity of GST H sites

B[a]P induced active H sites of several enzymes including GSTA2, GSTM1, GSTM2, GSTM4, GSTM6, and GSTP1. Induction with fold change over 2 was only observed in the highest 180 $\mu\text{mol/kg}$ B[a]P dose. In addition, GSTA4 showed significant 2.31-fold downregulation at the lowest dose (2 $\mu\text{mol/kg}$), 3.83-fold downregulation at 20 $\mu\text{mol/kg}$, and 5.13-fold downregulation at 180 $\mu\text{mol/kg}$ B[a]P (Figure 2, Table S1, Figure S3).

In comparison to the highly conserved G sites of individual GST isoenzymes, the substrate binding H sites are much more variable in sequence. The H sites of GSTs are generally electrophilic and/or hydrophobic and attract a wide range of substrates. The variability of GST H sites is thought to provide relative specificity of certain subtypes of xenobiotic

substrates. For instance, the alpha and mu classes of human cytosolic GSTs contain a dominantly hydrophobic H site while the enzymes in the pi class contain a half hydrophobic, half hydrophilic H site.⁸ As the substrate specificity of GSTs comes from distinct and variable H sites, post-translational modification of GSTs could feasibly result in the tuning of H sites for increased affinity toward particular substrates. ABPP using GSTABP-H can be used to discover expression independent induction of active H sites by comparing changes in GSTABP-H enrichment with changes in expression (via global proteomics).

B[a]P induction of GSTs as measured by analysis of the global proteome

Induction of protein expression was measured via global proteomics of unenriched liver cytosolic lysate. Linear regression and fold change analysis of B[a]P induction revealed significant increases in GSTA2, GSTM4, and GSTP1 enzymes. GSTA2 was induced by 3.1-fold, GSTM4 by 2.4-fold, and GSTP1 by 2.7-fold at 180 μ mol/kg (Figure 2, Table S1, Figure S3).

GST expression is known to be induced by Nrf2/Keap⁴⁵, pregnane X receptor (PXR)⁴⁶, and constitutive androstane receptor (CAR)⁴⁷ mechanisms. Nrf2/Keap1 is primarily involved in response to oxidative stress. Keap1 attenuates Nrf2 activation via ubiquitination, leading to degradation of Nrf2. In oxidative stress conditions or in the presence of electrophiles, a conformational change occurs in which Keap1 is deactivated, allowing active Nrf2 translocation to the nucleus and subsequently bind the antioxidant response element and elicit an inductive response.⁴⁸ PXR is a highly important receptor that aids in xenobiotic defense. This receptor is activated by a wide range of hydrophobic substances including toxins, bile acids, antibiotics, prescription drugs, and other chemicals.⁴⁹ CAR is a receptor that regulates the expression of many genes involved in all three phases of xenobiotic metabolism including oxygenation, conjugation, and excretion. Like PXR, CAR binds and is activated by a wide variety of hydrophobic endo- and xeno-biotics.⁵⁰ Because of the overlap between these pathways, it is possible that induction of GST expression following B[a]P exposure may be a result of a combination of direct binding of B[a]P and its downstream metabolites to these receptors as well as an oxidative stress response by Nrf2/Keap1.

Correlative analysis of induced changes in active GSTs versus GST protein expression

Following the determination of the specific GSTs induced (or inhibited) after exposure to B[a]P, we investigated the source of induction via comparison of induced or inhibited enzymes between datasets. Overall, we found that when comparing the B[a]P induction of the three proteomic datasets, three major groups of induction were revealed: (1) enzymes with B[a]P induced expression, *active* G sites, and *active* H sites, (2) enzymes with increases in active H sites with no expressional or G site induction, and (3) enzymes with reduced *active* G and H sites with no corresponding changes in expression.

GSTs with increased expression, G and H site activities

Enzymes whose induction was measured across all proteomic analyses include GSTA2, GSTM4, and GSTP1. All of these enzymes were found to show significantly induced expression (via global proteomics), and probe-determined G and H site activities based on fold change (≥ 2) and linear regression analysis ($p < 0.05$) (Figure 2, Figure 3A). For GSTP1,

results of the Pearson analysis reveal significant ($p < 0.05$) correlation between proteomics results from each individual probe and global samples. GSTP1 has previously been found to glutathionylate PAH diol epoxides.¹³ Knocking out this enzyme also has been shown to result in increased PAH-induced skin tumorigenesis in mice.⁵¹ Thus, increased GSTP1 activity as well as expression induced by PAH exposure may result in increased metabolism and detoxification of PAHs. GSTM4 induction was correlated between all proteomics datasets (Figure 3A). GSTM4 has not been previously shown to be induced by PAHs; nor has M4 been implicated in the metabolism of P450-bioactivated PAH metabolites.

Further studies are needed to determine the implications of induction of GSTM4 for PAH metabolism. A significant positive correlation between global and G site data of GSTA2 was observed (Figure 3A). GSTA2 is also previously known to show induced expression in rat hepatoma cells with PAH exposure,⁵² however little is known regarding its role in the metabolism and expulsion of bioactivated PAH metabolites. Further studies measuring PAH diol and diol epoxide metabolism should be conducted to determine its role. The similarity in overall inductive trends seen suggest that induction of *active* G and H sites is likely genetically derived and may constitute a receptor-mediated GST response.

GSTs with expression-independent increases activity unique to the H site

Three mu class GSTs (M1, M2, and M6) showed expression-independent increases in H site activity at 180 $\mu\text{mol/kg}$ B[a]P dose. Interestingly, not only do these enzymes show no increase in protein expression, they also show no increase in G site labeling by **GSTABP-G**. Correlation analyses reveal no correlation between expression and probe-determined G and H site activities for GSTM1. While expression, active G sites, active H sites are all positively correlated for GSTM2, only probe-determined H site activity significantly increases with dose (from fold change and linear regression analysis). Similarly, while global and **GSTABP-H** GSTM6 data show a positive correlation, fold change and linear regression analyses indicate that this enzyme shows significant dose dependence solely in GSTABP-H enriched samples and not in global samples (Figure 3B). These expression- and G site-independent increases in active H sites may suggest a post-translational mechanism that does not affect the ability of glutathione to bind the enzymes, but only increases the activity of H sites. We speculate that this post-translational mechanism may cause a conformational change in relation to the catalytic tyrosine residues responsible for stabilizing the glutathione thiolate anion formed in GST active sites. Several GST mu isoenzymes are also known to metabolize PAH diol epoxides.⁵³ In V79 cells, overexpression of GSTM1 resulted in a reduction of DNA adduct formation by dibenzo[a,h]pyrene and B[a]P diol epoxides.⁵⁴ In BPDE exposed H1355 cells, DNA damage was reduced in cells with exogenously overexpressed GSTM2.^{55, 56} Due to known roles in the metabolism of the prototypical PAH diol epoxides and prevention of DNA adducts in mammalian cells, this observed GSTM induction may prove important as traditional mRNA and global proteomics would not detect these changes.

GSTA4 expression-independent inhibition of G and H site activities

The enzyme most affected by B[a]P exposure was GSTA4, with significant downregulation observed at the lowest dose tested. **GSTABP-H** enriched A4 showed over 2-fold

downregulation at 2 $\mu\text{mol/kg}$ B[a]P and roughly 4- and 5-fold downregulation at 20 and 180 $\mu\text{mol/kg}$ doses. **GSTABP-G** enrichment of this enzyme only showed significant downregulation at the 180 $\mu\text{mol/kg}$ dose, while no changes in protein expression were observed. Pearson correlation analysis results showed no significant correlation between any of the three datasets though data from both probes seem to follow a similar trend, albeit at higher doses for **GSTABP-G** (Figure 3C). This could be suggestive of an inhibitory mechanism or post translational modification. GSTA4 is primarily known to be involved in metabolism of 4-hydroxynonenals and products of lipid peroxidation.⁵⁷ In addition, increased steady-state levels of aldehydes and decreased ability to conjugate 4-HNE have been observed in GSTA4 null mice.⁵⁸ The observed reduction in *active* G and H sites of this enzyme in the liver may lead to an impairment of the liver to detoxify these lipid derived free radicals and could equate to increased oxidative stress.

Conclusions

Exposure to xenobiotics, including PAHs, are characterized by the response of biotransformation enzymes in target tissues. Interindividual differences in detoxifying enzyme family expression and activity is principal to individual susceptibilities that result in PAH-induced toxicities and disease pathology. Our understanding of the oxidative response to PAH exposure is fairly well characterized at the expression and activity levels, particularly in the liver. In contrast, there is little isoenzyme specific activity and expression data for PAH induction of GSTs in mammalian model systems. Thus, in combination with the near complete lack of proteomics data for PAH induction of GSTs and the lack of enzyme-specific activity assays, our investigation improves our understanding of the role of individual GSTs in catalyzing glutathione conjugation in the metabolism of B[a]P.

According to the results described herein, the induction of active G and H sites and expression is moderate. However, large fold change GST induction may not be necessary for the metabolism of the oxidized B[a]P metabolites. Cytochrome P450 enzymes not only catalyze the initial oxidation of PAHs, they also are known to participate in further oxygenation of their products. Products of both P450 monooxygenation and epoxide hydrolase activities result in a much more complex mixture of B[a]P metabolites for conjugating enzymes to detoxify and metabolize.⁵ GSTs are thought to primarily catalyze the glutathione conjugation of PAH epoxides, diol epoxides, and diones while UGTs and sulfotransferases are thought to primarily catalyze the conjugation of glucuronic acid to PAH diols, for example.⁵ Thus, GSTs and other conjugating enzymes are likely responsible for the metabolism of small enzyme-family specific subsets of these complex metabolite mixtures. For this reason, doubling or tripling their expression and/or activity, resulting in a matching increase of metabolic capacity, may be ample for their metabolic roles and result in biologically significant increases in detoxification.

Our study reveals B[a]P induced GST activity and provides insight into subsite specific induction of GST activity. Intriguing differences are observed when comparing glutathione-binding and active H sites of the GSTs in this study. At high doses of B[a]P, probe-determined H site activities of three GST mu enzymes show both expression and glutathione binding- independent increases. It is also conceivable that upon certain perturbations,

glutathione-binding sites may also show expression-independent activity changes. These findings may generate hypotheses for further investigation into potential post-translational mechanisms of this site-specific induction of activity. In addition, further investigations as to the effect on the rate of GST metabolism of oxidized benzo[a]pyrene metabolites in induced animal models should be conducted in order to determine the role of induced GST enzyme activities in the detoxification of these carcinogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ABPP	activity-based protein profiling
ABPs	activity-based probes
Ahr	aryl hydrocarbon receptor
AMT	accurate mass and time
B[a]P	benzo[a]pyrene
BCA	bicinchoninic assay
BPDE	benzo[a]pyrene diol epoxide
CAR	constitutive androstane receptor
DMSO	dimethylsulfoxide
DNCB 2	4-dinitrochlorobenzene
GSH	glutathione
GST	glutathione S-transferase
LC-MS	liquid chromatography – mass spectrometry
MT FDR	mass and time false discovery rate
Nrf2	nuclear factor-like 2
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline

PXR pregnane X receptor

SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Figure 1.

(A) GSTABP-G and **(B) GSTABP-H** labeling of liver cytosol from mice exposed to water vehicle, corn oil vehicle, 2, 20, or 180 μmol/kg B[a]P. The fluorophore TAMRA was attached to probe-labeled proteins via click and the proteins were separated by electrophoresis and visualized and quantified by fluorescence. The prominent band at roughly 25 kDa (GST molecular weight) was quantified using ImageJ software and charted as the upper bar graphs; values are depicted relative to corn oil vehicle. Error bars represent 95% CI of the average of three technical replicates.

Figure 2.

Global proteomics and ABPP results as depicted by volcano plots. Log₂ fold changes of relative abundances of GSTs in 180 μmol/kg B[a]P vs. corn oil vehicle samples are plotted on the x axes. -log₁₀ *p*-values from linear regression of all samples/conditions are plotted on the y axes. Dotted lines are located at *p* = 0.05 and log₂ fold change = 1. Thus, values in the upper right and left quadrants are GSTs that are significantly induced by B[a]P treatment. Analyses are from 3 technical replicates from pooled cytosolic samples from 4 individually exposed mice.

Figure 3.

Fold change and correlation analyses of GSTs for which significant induction occurred upon B[a]P exposure. For each GST enzyme, fold change induction relative to corn oil vehicle samples is shown (top) as well as results from Pearson Correlation analysis results including matrices of correlation coefficients (r) and p values for indicated comparisons. These correlation comparisons include global vs. GSTABP-G, global vs. GSTABP-H, and GSTABP-G vs. GSTABP-H. (A) Fold change and correlation analysis results of GST enzymes with significant induction as measured by global, **GSTABP-G** enriched, and **GSTABP-H** enriched proteomics samples (B) Fold change and correlation analysis results

of GST enzymes with significant induction as measured by **GSTABP-H** enriched proteomics samples, but not global nor **GSTABP-G** enriched samples (C) Fold change and correlation analysis results of GSTA4, the sole GST in which B[a]P exposure resulted in changes in activity as measured by both probes, with no corresponding changes in expression as measured by global proteomics. Analyses are from 3 technical replicates from pooled cytosolic samples from 4 individually exposed mice. Error bars represent standard error of the mean.

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Table 1.

Enrichment (probe vs. DMSO only) of GSTs in liver cytosols from mice treated with B[a]P and vehicle controls

	GSTABP-G		GSTABP-H	
Protein	Fold Change Enrichment	<i>p</i> value (t-test)	Fold Change Enrichment	<i>p</i> value (t-test)
GSTA2	53.1	1.67E-02	116.2	8.73E-03
GSTA3	43.7	1.86E-15	141.9	6.88E-16
GSTA4	48.6	1.82E-11	24.7	1.63E-08
GSTK1	8.4	6.15E-13	72.0	1.74E-20
GSTM1	234.4	1.53E-15	164.4	1.21E-12
GSTM2	131.7	1.96E-13	45.9	6.67E-10
GSTM4	93.5	3.12E-13	1251.8	3.61E-16
GSTM5	8.2	5.08E-10	370.7	2.84E-16
GSTM6	29.6	6.14E-07	171.5	6.66E-08
GSTM7	40.4	2.92E-10	61.7	3.76E-10
GSTO1	3.8	3.38E-09	84.3	3.89E-15
GSTP1	19.2	1.88E-10	699.7	7.07E-16
GSTT1	10.2	4.86E-09	545.2	3.56E-15
GSTT2	6.3	5.61E-05	1400.9	4.29E-13