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Expression of glutathione S-transferases in fetal lung and liver tissue from parental strains and F1 crosses between C57BL/6 and BALB/c F1 mice following in utero exposure to 3-methylcholanthrene

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AHH, aryl hydrocarbon hydroxylase activity
CDNB, 1-chloro-2, 4-dinitrobenzene
Cyp, cytochrome P-450
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GST, glutathione S-transferase

ABSTRACT

GST isoforms have been extensively studied in adult tissues but little is known about the composition and levels of these enzymes in fetal tissues. As part of our ongoing studies to determine the potential role of metabolic enzymes in mediating the differential susceptibility of different strains of mice to lung tumorigenesis following in utero exposure to 3-methylcholanthrene (MC), we screened for GST enzyme activity and for expression of the individual *GST*α, π, μ, and θ isoforms in murine fetal lung and liver tissues isolated from the parental strains and F1 crosses between C57BL/6 (B6) and BALB/c (C) mice. Using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, we found that treatment with MC had no effect on the levels of GST enzyme activity in either the fetal lung or liver in either of the two parental strains or their F1 crosses. Low levels of expression of each of the four enzymes were detected by Western blotting in both fetal lung and liver tissues in all four strains. A statistically significant 3.5-fold induction was observed only for *GST*μ in the fetal lung of the parental strain of BALB/c mice 48 h after exposure to MC. None of the other enzymes showed any significant differences in the levels of expression following exposure to MC. Although strain-specific differences in the expression of the GSTs that were independent of MC treatment were observed, they could not account for the differences previously observed in either the Ki-*ras* mutational spectrum or lung tumor incidence in the different strains of mice. Similar results were obtained when the maternal metabolism of MC was assayed in liver microsomal preparations. The results are consistent with previous studies showing low levels and poor inducibility of phase II enzymes during gestation, and demonstrate for the first time that all four of the major *GST* enzymes are expressed in fetal tissues. While the

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MC, 3-methylcholanthrene
NAT, *N*-acetyltransferases
PAH, polycyclic aromatic hydrocarbons
Pas, pulmonary adenoma susceptibility
UDPGT, uridine 5′-diphosphoglucuronosyltransferase

high inducibility of activating enzymes, such as *Cyp1a1*, and low, uninducible levels of phase II conjugating enzymes probably account for the high susceptibility of the fetus to transplacentally induced tumor formation, the results also suggest that factors other than metabolism may account for the strain-specific differences in susceptibility to carcinogen-mediated lung tumor induction following in utero exposure to chemical carcinogens.

1. Introduction

Studies from several laboratories have provided evidence that the fetus and embryo are more susceptible to environmental toxicants than are adult organisms [1–6]. One of the factors that appears to mediate the higher susceptibility of the developing organism to environmental carcinogens is the difference in composition and concentration of metabolic enzymes responsible for the activation and/or detoxification of carcinogenic agents. In general, fetal tissues have low levels of drug metabolic enzymes and lack many of the forms of phase I enzymes present in adult tissues [4,7–10]. In addition, the fetus exhibits low levels and rather poor inducibility for many of the protective phase II enzymes [5,11,12]. Thus, when exposed in utero to chemical carcinogens such as PAHs, the fetus demonstrates a marked induction of *Cyp1a1*, resulting in the metabolic activation of the parent PAH to a highly reactive electrophile capable of binding to DNA and forming potentially mutagenic DNA adducts. Since this marked induction of *Cyp1a1* occurs in the relative absence of other forms of Cyp that detoxify the PAH – and because of the poor inducibility of the phase II enzymes – the fetus demonstrates a higher relative induction of AHH activity compared to adult tissues [5,13–16].

Our laboratory has compared the effect of in utero exposure to MC on lung tumor incidences in D2 × B6D2F1 backcrossed mice and the more susceptible BALB/c strain [17,18]. Interestingly, we found that BALB/c mice had a reduced latency for lung tumor formation. All of the BALB/c mice treated in utero with a 45 mg/kg dose of MC had lung tumors 6 months after birth, while the *Cyp* inducible D2 × B6D2F1 backcrossed mice treated in utero with 30 mg/kg of MC had a tumor incidence of 84% after 12 months. The two strains also demonstrated differences in the mutational spectrum of the Ki-*ras* gene, as D2 × B6D2F1 backcrossed mice exhibited G → T transversions in 84% of their lung lesions while BALB/c mice exhibited a predominance of G → C transversions in 62% of the lung lesions with Ki-*ras* mutations [17,19,20]. Recent studies from our laboratory have shown that the differences in mutational spectrum induced in the Ki-*ras* gene were not due to differences in the levels of Phase I enzymes expressed during late gestation [21].

Although the identification and determination of the levels and composition of phase I enzymes has received a great deal of attention, much less is known about the levels and activity of phase II enzymes during gestation [12]. The majority of studies have been conducted in liver tissue, with little or no information available on other organ sites. Glutathione *S*-transferases (GSTs) are a family of enzymes that catalyze the conjugation of glutathione to reactive electrophiles [22–24]. In studies directly comparing fetal to adult tissue, Di Ilio et al. [25], using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, demonstrated GST activity in fetal mice on the 14th and 18th days of gestation. Similar to results obtained with phase I enzymes, they showed that fetal liver activity in untreated mice was approximately 35% of the levels found in adult livers, although the inducibility of fetal GST activity by PAHs was not determined. Previous studies from our laboratory also demonstrated low levels of CDNB activity during late gestation in fetal lung and liver supernatants from C57BL/6 mice [26]. We found that the fetal liver tissue exhibited approximately seven-fold higher levels of CDNB activity than did fetal lungs, and also demonstrated expression of GSTα by Western blotting in both organs. Hales and Huang [27] demonstrated the expression of the Y_p subunit of *GSTP* in the yolk sac of rat embyros during organogenesis. In addition, human fetal livers were shown to contain low levels of GST expression in conjunction with a decreased ability to detoxify hydroperoxides [28].

It has been shown in adult animals that MC can be conjugated by *GST* enzymes [29]. Thus, in order to determine the potential role of GSTs in the strain-specific differences observed in the Ki-*ras* mutational spectrum following in utero exposure to MC, and to determine the composition and levels of the different GSTs during gestation in potential target tissues for PAH-mediated carcinogenesis, we examined fetal lung and liver tissues for expression of the four major enzymes of *GST* in C57BL/6, BALB/c, and reciprocal crosses between these two strains of mice. We sought to determine if differences in the ability of different strains of fetal mice to detoxify reactive electrophiles could account for differences in their susceptibility to tumor formation or the mutational spectrum of Ki-*ras* mutations.

2. Materials and methods

2.1. Animals and treatment protocols

BALB/c (C) and C57BL/6 (B6) mice were obtained from Charles River Laboratories (Raleigh, NC). The mice were housed in a pathogen-free environment in plastic cages with corn cob bedding and aspen pile for nesting. The mice were allowed free access to food (Prolab RMH 3000, Lab Diet, Richmond, IN) and water, and were maintained on a 12 h fluorescent light/dark cycle. Mice were mated by placing one female and one male together overnight in a cage. Pregnant mothers were treated on the 17th day of gestation (day 1 was the first day

following overnight mating) by a high i.p. injection under the diaphragm with either 0.5 ml/0.035 kg body weight of olive oil or a 45 mg/kg dose of MC dissolved in olive oil. Mice were euthanized by CO_2 asphyxiation followed by exsanguination at various time points as described below. All animal studies were approved by the Institutional Animal Care and Use Committee of the Wake Forest University School of Medicine.

2.2. Determination of GST enzyme activity

Pregnant mothers were euthanized by CO_2 asphyxiation/exsanguination at 8, 24, and 48 h after injection. Maternal livers and fetal lung and liver tissues were collected separately and flash frozen in liquid nitrogen immediately upon dissection and stored at −80 °C (pooled fetal lung or liver tissue from three individual litters were isolated for each time point). Fetal lung and liver tissues were homogenized in cold 0.1 M potassium phosphate buffer (pH 7.25) and the homogenates then subjected to three cycles of freezing in liquid nitrogen and thawing in a room temperature water bath. Supernatant fractions were isolated by centrifugation for 10 min at 14,000 rpm at 4 °C. Protein content was determined with the Bio-Rad Protein Assay Kit (Hercules, CA).

GST enzyme activity was assayed by a modification [30] of the method described by Habig et al. [31]. Briefly, 10–20 μg of protein was assayed at 23 °C in a solution of 0.1 M potassium phosphate (pH 6.5) and 1 mM glutathione. The reaction was initiated by addition of 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in 20× ethanolic solution. Change in absorbance was monitored at 340 nm for 90 s. Activity was calculated using the ΔA/min and extinction coefficient and reported as nmol/min/mg protein.

2.3. Determination of GST protein expression level by Western blot

As previous studies from our laboratory have shown that the GST specific activity in clonal GST-transfected cell lines is proportional to the band intensity by Western blot [32], we utilized Western blotting to determine the levels of the different GST enzymes. Fetal lung and liver tissues (tissues from individual litters were pooled and there were three litters for each time point) were homogenized in cold lysis buffer (25 mM Tris–HCl, pH 7.4/150 mM NaCl/1% Triton X-100/1% SDS) containing a cocktail of protease inhibitors (*Complete*, Roche, Indianapolis, IN) and phenylmethanesulfonyl fluoride (0.1 mg/ml). Equal amounts of protein from each lysate were separated on 12% SDS-PAGEs and analyzed by Western blotting using a 1:1000 dilution of affinity-purified, class-specific rabbit anti-GST polyclonal antibodies developed against purified human α, μ, or π class GSTs [33], and a monoclonal mouse antibody for GSTθ (clone GSTE1-1A2, provided as a gift from LabAs Ltd. Tartu, Estonia). Secondary antibodies consisted of a 1:10,000 dilution of either goat anti-rabbit or goat anti-mouse (for GSTθ) IgG conjugated to horseradish peroxidase (ICN/Cappel, Aurora, OH). Bands were visualized using enhanced chemiluminescence according to the manufacturer's instructions (Bio-Rad). Equivalent loading and protein transfer were confirmed by Western blot using monoclonal mouse anti-rabbit GAPDH antibody (RDI, Flanders, NJ) and by staining membranes with 1% of Ponceau S (Sigma, St. Louis, MO). Films were scanned with a scanner (EPSON TWAIN 5, version 5.71A) and band densities were analyzed using the UN-SCANIT Automatic digitizing system (Version 5.1). GST values at each time point were expressed relative to the levels of GAPDH expression.

2.4. Determination of MC metabolism in maternal liver

The frozen maternal livers from the pregnant mothers were thawed on ice and rinsed in 0.25 M potassium phosphate, pH 7.25/0.15 M KCl buffer. The tissues were homogenized in 10 volumes (w/v) of buffer and centrifuged at 9000 × *g* for 20 min at 4 °C in an Eppendorf 5804R centrifuge using a fixed angle rotor. The supernatants were then centrifuged at 42,000 rpm for 60 min at 4 °C in a Beckman 70.1Ti rotor and the pellet resuspended in 0.25 M K-PO_4 (pH 7.25)/30% glycerol and the samples stored at −80 °C until use. Protein content was determined with the Bio-Rad Protein Assay Kit.

For determination of MC metabolism, the reaction mixtures contained 100 μl of 1 M potassium phosphate buffer (pH 7.4), 50 μl of 100 mM magnesium chloride, 100 μl of 0.2 M glucose-6-phosphate (in 40 mM Tris–HCl buffer, pH 7.3), 20 μl of 50 units/μl of glucose-6-phosphate dehydrogenase (in 5 mM sodium citrate buffer, pH 7.5), 100 μl of 6 mg/ml BSA, 50 μl of 10 mM β-nicotinamide adenine dinucleotide phosphate, and 100 μl of 4 mg/ml of liver microsomes diluted in 100 mM potassium phosphate buffer (pH 7.4). The reactions were started by the addition of 10 μl of 8 mM MC (in acetone) and were incubated at 37 °C in a shaking water bath for 7.5 min. The reactions were quenched by the addition of 1 ml of ethyl acetate and the samples chilled on ice. The reactions were done in duplicate for each of three liver microsomal protein samples.

The reaction mixtures were extracted three times by the addition of 2 ml of ethyl acetate. Each sample was vortexed, centrifuged, and the upper layer removed to a glass culture tube for a final extract volume of approximately 6 ml. The ethyl acetate was removed by rotary evaporation and the samples dissolved in 100 μl of methanol.

A 20 μl sample was injected onto an Agilent 1100 series LC/MSD single quadrapol system in atmospheric chemical ionization (APCI) mode. Separations were carried out on a 5 μm Agilent Zorbax SB-C18 column (4.6 mm × 150 mm) at 25 °C with a flow rate of 0.75 ml/min. The mobile phase was a binary gradient of 0.1% formic acid in deionized water (solvent A) and 0.1% formic acid in acetonitrile (solvent B): a linear gradient from 40% solvent A and 60% solvent B to 10% solvent A and 90% solvent B at 20 min, isocratic at 10% solvent A and 90% solvent B from 20 to 40 min, then a linear gradient to 100% solvent B at 50 min, and hold for 10 min. Each run was followed by a 15 min equilibration time at initial conditions.

Data were collected with an Agilent 1100 series PDA at 295 nm and 1100 series MSD. The MSD source was operated in positive APCI mode with a corona current of 4000 nA, the vaporizer at 400 °C, a gas temperature of 350 °C with a flow of 5 l/min, and a nebulizer pressure of 60 psi. The MSD collected data in single ion monitoring (SIM) mode for metabolite fragments of masses 267, 283, and 299 *m/z*.

2.5. Statistical methods

2.5.1. Fetal levels of GSTs

Statistical analyses were performed to (1) assess if significant differences existed between the olive oil controls and the 24 h and/or 48 h MC treated samples for each liver and lung combination across each of the two parental strains and their F1 crosses, and (2) compare these four strains for significant differences for each group combination for 24 h olive oil control, 24 h MC, and 48 h MC treatment. Descriptive analyses were performed to explore distribution shape and characteristics of GST levels. A logarithmic transformation was used in the analysis to stabilize variation and preserve normality.

For comparison of the 24 h MC and 48 h MC treatments to the olive oil control, *t*-tests were performed comparing ln(GST). Results were adjusted for multiple comparisons using stepwise Bonferroni type adjustments [34,35]. For comparison of the four strains for each treatment, Analysis of Variance was performed with ln(GST) for the response and strain.

2.5.2. Maternal metabolism of MC

Descriptive statistics were examined for assay levels by metabolite to assess their distribution shape and characteristics. Analysis of Variance (ANOVA) was performed on the levels to assess strain and treatment group differences in enzyme activity for each metabolite. Tests of interaction between strain and treatment group were performed. If significant, tests of simple effects for strain differences within treatment group and treatment group differences within strain were performed. Adequacy of model assumptions was examined by analysis of residuals. For the 1-one and 2-OH metabolites, a log transformation was used to preserve normality after such analysis indicated some inadequacy. All analyses were performed using SAS v8.2 (SAS Institute, Cary, NC).

3. Results

GST isoforms have been extensively studied in adult tissues in both humans and mice. However, little is known about the composition and levels of these enzymes in fetal tissues. As part of our ongoing studies to determine the potential role of metabolic enzymes in mediating the differential susceptibility of different strains of mice to lung tumorigenesis following in utero exposure to MC, we screened for GST enzyme activity and for expression of the individual *GST*α, π, μ, and θ isoforms in murine fetal lung and liver tissues isolated from C57BL/6 (B6), BALB/c (C), and F1 crosses between the two strains of mice. Pregnant mothers were treated on the 17th day of gestation with either olive oil alone or 45 mg/kg of MC dissolved in olive oil. Fetal tissues were collected at 8, 24 and 48 h after injection.

Using CDNB as a substrate, we found that treatment with MC had no effect on the levels of GST enzyme activity in either the fetal lung (Fig. 1A) or liver (Fig. 1B) in either of the two parental strains and their F1 crosses. These results confirmed previous studies conducted by Di Ilio et al. [25] and our laboratory [26]. We then used Western blotting to determine the expression of the four major GSTs in fetal lung and liver

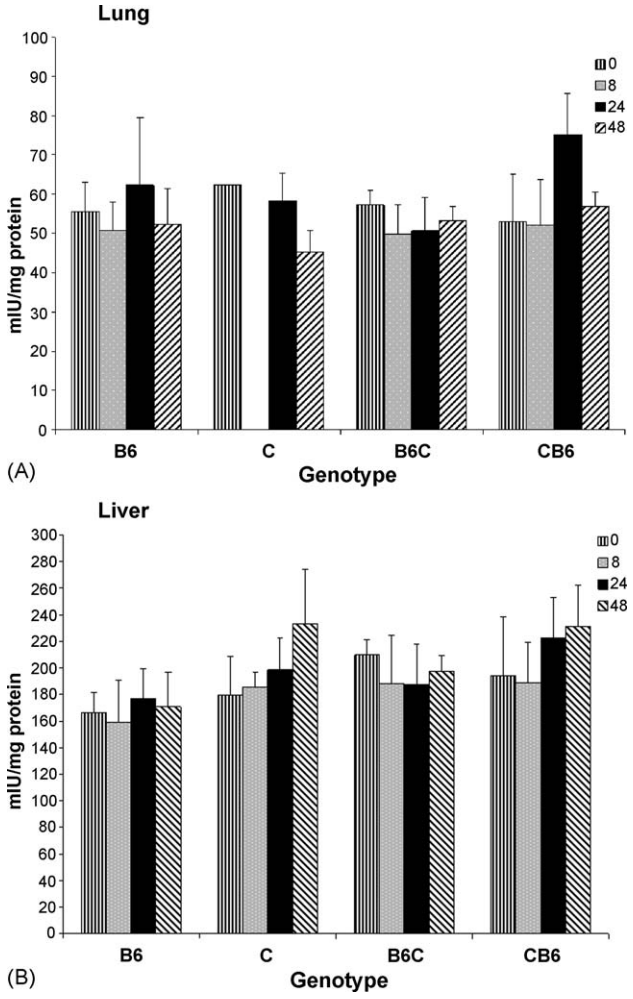
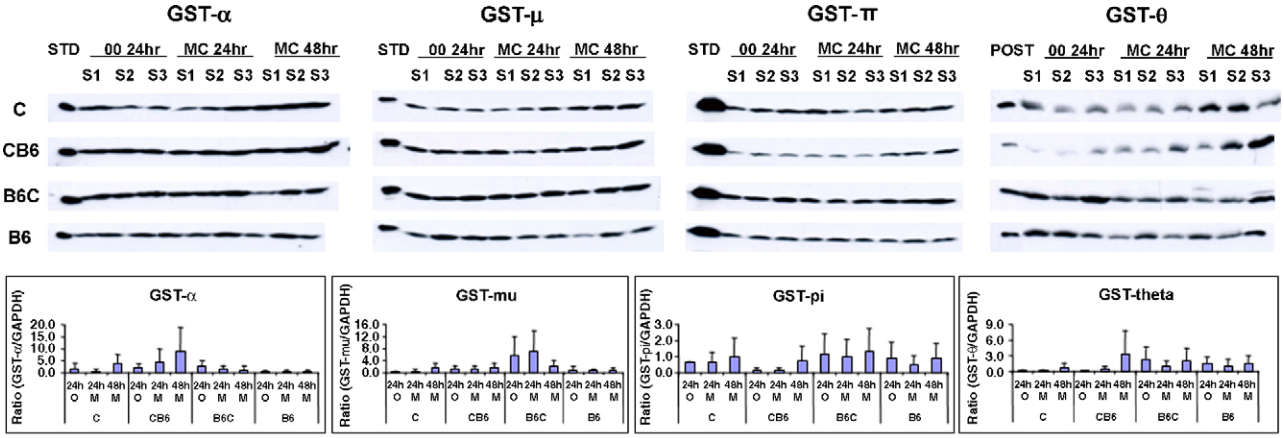


Fig. 1 – GST enzyme activity in fetal tissues. Fetal lung and liver tissues were collected 8, 24, and 48 h after injection of MC. GST enzyme activity was assayed using 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Activity was calculated using the ΔA/min and extinction coefficient, reported in nmol/min/mg protein. 0: control samples collected 24 h after injection with the olive oil vehicle. For the histogram, each bar represents the mean ± S.D. of three individual litters. It should be noted that the 8 h fetal lung samples for C mice were lost due to a technical error.

tissues in the different mouse strains (Figs. 2 and 3). We were able to detect low levels of expression of each of the four GSTs in both the fetal lung and livers in all four strains. Because of the low levels of expression and the uncertainty in determining the exact time of conception, a high degree of variability was seen between individual litters in the same treatment group. *GST*θ, in particular, showed a very high degree of inter-sample variation, particularly in the lung (Fig. 2). Statistical analyses indicated that the only significant difference detected following in utero exposure to MC was a significant increase in the levels of *GST*μ in the fetal lung of the parental strain of BALB/c mice in lung tissue isolated 48 h after in utero exposure to MC, which increased by 3.5-fold at this time point relative to the olive oil treated group ($P = 0.01$). At all other time



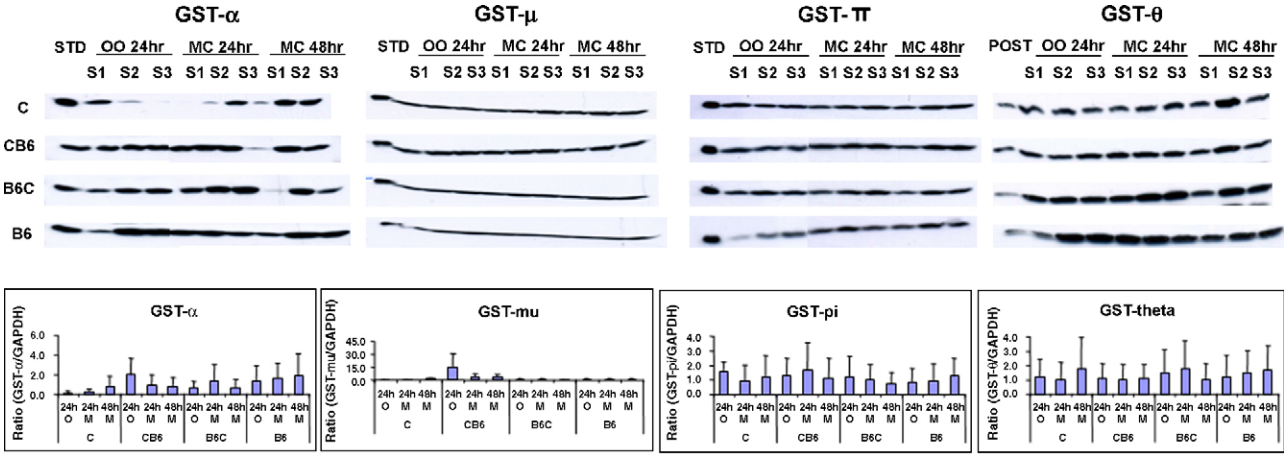
GST Isozymes Fold Induction in Fetal Lung Tissues												
	C			CB6			B6C			B6		
fold induction	24hr OO	24hr MC	48hr MC	24hr OO	24hr MC	48hr MC	24hr OO	24hr MC	48hr MC	24hr OO	24hr MC	48hr MC
GST-α	1.00	0.36	2.39	1.00	2.34	4.64	1.00	0.49	0.44	1.00	1.38	1.28
GST-mu	1.00	1.12	3.50	1.00	1.31	1.47	1.00	1.19	0.32	1.00	0.63	0.76
GST-pi	1.00	0.99	1.62	1.00	1.00	5.42	1.00	0.86	1.09	1.00	0.56	0.96
GST-theta	1.00	0.55	4.39	1.00	6.24	48.07	1.00	0.42	0.85	1.00	0.73	0.97

Fig. 2 – Western blot analysis of GSTα, μ, π and θ protein in fetal lung following transplacental exposure to MC. Pregnant mothers were treated on day 17 of gestation with either olive oil (O) or 45 mg/kg of MC (M). Mice were euthanized by CO_2 asphyxiation/exsanguination and homogenates prepared from fetal lung tissues 24 and 48 h after injection. Lungs from the same litter were pooled. Tissues homogenates and protein standards (STD) or positive controls from transfected cell lines (POST) were electrophoresed on 12% SDS PAGEs, transferred to nitrocellulose membranes, and the membranes blotted with antibodies to the different GSTs. Three litters are shown for each time point. The results from the Western blots shown in the upper part of the figure are also presented as a histogram showing densitometric values in arbitrary units. For the histogram, each bar represents the mean ± S.D. of three individual litters. Values in the histogram are expressed as the ratio of the value obtained for GST divided by the value obtained for expression of GAPDH (densitometric analysis of GST expression relative to GAPDH). These data are presented as fold induction over 24 h olive oil treated controls in the table at the bottom of the figure.

points in both organs, none of the other GSTs showed any significant differences in the levels of expression following exposure to MC. To be sure that developmental alterations in the levels of the individual enzymes did not occur from gestation day 18 to 19, which could impact our analysis of enzyme induction relative to the 24 h control, we also compared the 24 h olive oil treated controls to another group of mice euthanized after a 48 h exposure to olive oil. Homogenates from three individual litters were pooled and analyzed by Western blotting (Fig. 4). The results showed that the basal levels of each GST were stable over the 24 and 48 h post-injection time period in which the samples were assayed for induction by MC. The results are consistent with previous studies showing low levels and poor inducibility of phase II enzymes during gestation, and demonstrate for the first time that all four of the major GSTs are expressed in fetal tissues [12,25,26]. The mean values obtained for *GSTθ*, though not significant, would at first glance appear to suggest a rather marked induction of the enzyme following in utero exposure to MC, particularly in the C × B6 mice. However, careful inspection of the Western blots show that at both the 24 and 48 h post-injection time points, this increase is due mainly to one of the three samples analyzed.

There did appear to be some strain-specific differences in the expression of the GST enzymes that were independent of the treatment. Statistically significant differences ($P < 0.05$) were found across the four strains for GSTμ in the olive oil treated lung and liver groups, as well as in the 24 h MC treated liver. Statistically significant differences between expression in the four strains were also noted in the olive oil treated lung for expression of GSTπ and θ, and for GSTθ in the 24 h MC treated groups. However, no consistent patterns of differences in expression could be determined that would account for the differences in mutational spectrum observed in D2 × B6D2F1 backcrossed and BALB/c mice [17,19].

Finally, we also determined the ability of maternal liver to metabolize MC in order to ascertain if subtle differences in maternal metabolism could influence the amount of carcinogen available that could cross into the fetal compartment. Pregnant mice were treated with 45 mg/kg of MC on day 17 of gestation and liver microsomes were prepared from the maternal livers 24 h after injection of MC. As shown in Table 1, we measured 5 specific metabolic products of MC, including the 1-OH, 1-one, 2-OH, 11,12-diol, and 11,12-dione. We found statistically significant differences between each of the strains for a number of the metabolites produced following MC metabolism, both in the oil-treated control and MC-treated samples. Type III tests for significant interaction between strain and treatment group indicated that there were significant differences for all metabolites except for the



GST Isozymes Fold Induction in Fetal Liver Tissues

	C			CB6			B6C			B6		
fold induction	24hr OO	24hr MC	48hr MC	24hr OO	24hr MC	48hr MC	24hr OO	24hr MC	48hr MC	24hr OO	24hr MC	48hr MC
GST-α	1.00	1.76	5.88	1.00	0.48	0.37	1.00	2.19	1.06	1.00	1.29	1.48
GST-mu	1.00	0.75	1.69	1.00	0.23	0.21	1.00	0.76	0.68	1.00	0.98	1.23
GST-pi	1.00	0.59	0.78	1.00	1.26	0.87	1.00	0.82	0.60	1.00	1.14	1.52
GST-theta	1.00	0.81	1.42	1.00	0.97	0.99	1.00	1.21	0.69	1.00	1.24	1.37

Fig. 3 – Western blot analysis of GSTα, μ, π and θ protein in fetal liver following transplacental exposure to MC. Pregnant mothers were treated on day 17 of gestation with either olive oil (O) or 45 mg/kg of MC (M). Mice were euthanized by CO_2 asphyxiation/exsanguination and homogenates prepared from fetal liver tissues 24 and 48 h after injection. Livers from the same litter were pooled. Tissues homogenates and protein standards (STD) or positive controls from transfected cell lines (POST) were electrophoresed on 12% SDS PAGEs, transferred to nitrocellulose membranes, and the membranes blotted with antibodies to the different GSTs. Three litters were analyzed for each time point. The results from the Western blots shown in the upper part of the figure are also presented as a histogram showing densitometric values in arbitrary units. For the histogram, each bar represents the mean ± S.D. of three individual litters. Values in the histogram are expressed as the ratio of the value obtained for GST divided by the value obtained for expression of GAPDH (densitometric analysis of GST expression relative to GAPDH). These data are presented as fold induction over 24 h olive oil treated controls in the table at the bottom of the figure.

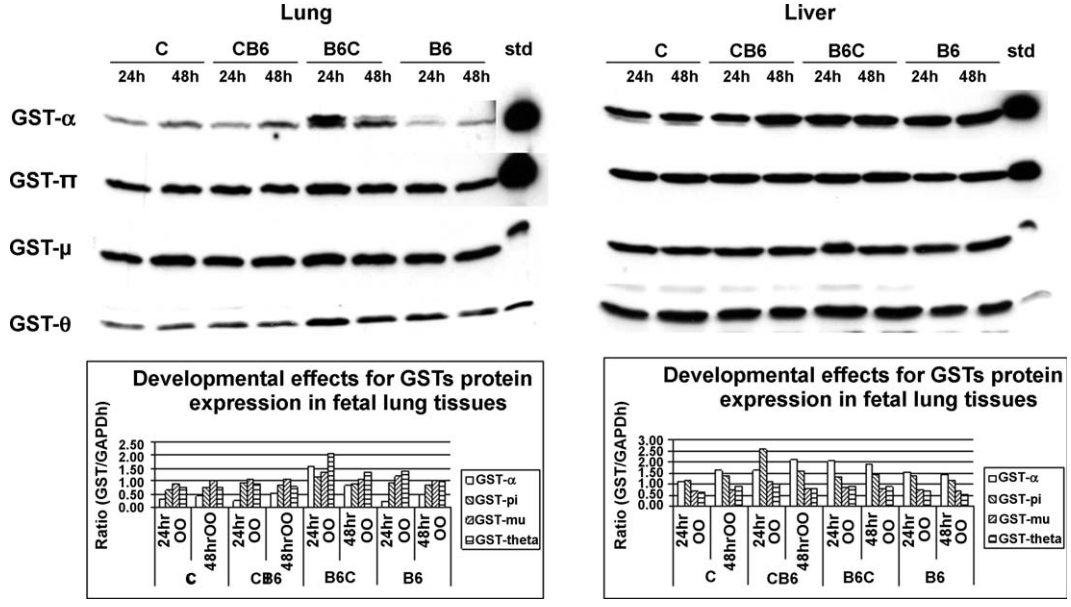


Fig. 4 – Basal levels of GST expression in fetal tissues on gestation days 18 and 19. Pregnant mothers were treated on day 17 of gestation with olive oil and euthanized by CO_2 asphyxiation/exsanguination. Homogenates were prepared from fetal lung and liver tissues 24 and 48 h after injection. Tissues homogenates from two to three individual litters were pooled. Tissues homogenates and protein standards (STD) were electrophoreses on 12% SDS PAGEs, transferred to nitrocellulose membranes, and the membranes blotted with GST antibodies. The data in the histogram are expressed as the ratio of GST/GAPDH (densitometric analysis of GST expression relative to GAPDH).

Table 1 – Metabolism of MC by maternal liver microsomes from different strains of mice

	B6			B6 × C			C × B6			C		
	Cont	MC	%Cont	Cont	MC	%Cont	Cont	MC	%Cont	Cont	MC	%Cont
1-OH	5.45 ± 0.28 c	4.60 ± 0.57 a	84	3.43 ± 0.43 d	4.79 ± 0.35 a	140[†]	6.34 ± 1.12 c	7.05 ± 0.91 b	111	3.97 ± 0.92 d	6.20 ± 0.79 b	156[a]
1-One	3.60 ± 1.13	4.02 ± 0.47	112	2.77 ± 0.28	3.79 ± 0.17	137	5.56 ± 1.37	7.43 ± 5.21	134	6.06 ± 1.10	8.56 ± 2.23	141
2-OH	3.04 ± 0.38	9.13 ± 1.76 a	300[†]	2.22 ± 0.30	7.93 ± 5.25 a	357[†]	2.09 ± 0.11	2.80 ± 0.82 b	134	2.00 ± 0.60	3.21 ± 0.44 b	161[a]
11,12-Diol	1.04 ± 0.90	6.61 ± 0.42 a	636[†]	ND	6.21 ± 2.61 a	–	ND	1.19 ± 2.07 b	–	0.49 ± 0.09	2.39 ± 2.07 b	488
11,12-Dione	0.19 ± 0.11	0.44 ± 0.04 a	232[†]	0.11 ± 0.06	0.45 ± 0.13 a	409[†]	0.26 ± 0.24	0.16 ± 0.08 b	62	0.13 ± 0.06	0.38 ± 0.09 a	292[a]

The concentration of metabolites of MC was determined by LC/MS following incubation of 400 μg of maternal liver microsomes with MC for 7.5 min. Values are expressed as nmol/min/mg protein. ND: not detected. For MC treated enzyme activity, strains with different letters differed significantly from each other by a minimum of $P < 0.05$ for each metabolite. For control enzyme activity, strains with different letters differed statistically from each other by a minimum of $P < 0.05$ for each metabolite. Type III tests for significant interaction between strain and treatment group indicated that there were no statistically significant differences for the 1-one metabolite in either the control or MC treated groups; no statistically significant differences were observed in the control groups for the 2-OH, 11,12-diol, and 11,12-dione as well. Values represent the mean ± S.D. for three maternal liver microsomal samples.

[†] Statistically significant induction in MC treated vs. control value for each metabolite in each strain from within strain ANOVA comparisons.

1-one. For this metabolite, there were significant differences between strains ($P = 0.0008$) and between the MC treated and control groups ($P = 0.06$). For the other four metabolites, there were significant strain differences in the control group only for the 1-OH metabolite ($P = 0.0006$). For the MC treated groups, strains were significantly different for these metabolites ($P < 0.025$). Differences within strain between control and MC-treated groups varied for these four metabolites, with B6 × C mice exhibiting significant differences for these metabolites ($P < 0.04$). Importantly, while pairwise comparisons between the strains demonstrated significant differences between specific strains for some of the metabolites, the B6 mice did not differ from each of the other three strains for any of the five metabolites of MC, suggesting that differences in maternal metabolism could not account for the observed differences in tumor incidence noted in our more recent studies in B6 and C mice [36]. Thus, similar to the results obtained previously for the Phase I enzymes and formation and repair of MC-DNA adducts in fetal tissue samples [21], we found that, although there were some differences in the amount of specific products formed across each of the four strains, none of these differences in the maternal metabolism of MC could account for the marked differences in tumor induction (Table 1).

4. Discussion

The results of these experiments demonstrate for the first time the expression of all four of the major GSTs in the late gestation mouse fetus, and that all four forms are expressed concurrently in the same tissues. Previous studies have shown that the fetus and embryo are more sensitive to chemical and physical carcinogens than are adult animals [1,5,6]. A number of studies have shown that fetal tissues exhibit relatively low levels of drug metabolic activity compared to adult tissues [4,7–9]. Thus, transplacental exposure to PAHs results in a very high level of *Cyp1a1* induction [5,10,13,37]. Using RNA isolated from the same tissues used in this study, we have shown that the levels of *Cyp1a1* transcripts were induced 7000- to 16,000-fold and 2000- to 6000-fold in fetal liver and lung tissue, respectively. Because of the poor inducibility of the phase II enzymes during development [5,11,12], this results in a very large increase in the relative activity of the major enzyme responsible for PAH metabolism, namely *Cyp1a1*, in the absence of an increase in protective detoxifying enzymes. In addition to the *GST*s, several studies have demonstrated low levels of uridine 5′-diphosphoglucuronosyltransferase activity in the developing fetus of several organisms [11,12], while McQueen et al. [38] have demonstrated low levels of *NAT1* and *NAT2* enzymatic activity and RNA transcripts, as well as reduced acetylation of 4-aminobiphenyl, in murine fetal liver relative to the activity observed in adult liver tissue. Thus, it appears that the high inducibility for induction of activating enzymes, such as *Cyp1a1*, and low, uninducible levels of phase II conjugating enzymes probably contribute to the high susceptibility of the fetus to transplacentally induced tumor formation. These differences in metabolic activity toward carcinogenic agents, combined with the high proliferative capacity of fetal tissues due to their high rate of growth, leaves

the fetus less time to repair damage to DNA prior to the next round of DNA replication and increases the likelihood of gene mutations occurring that lead to cancer and other diseases.

Recent studies from our laboratory have provided evidence suggesting that B6 mice are relatively resistant to the induction of lung cancer following in utero exposure to MC [36]. This finding was unexpected since 84% of inducible D2 × B6D2F1 backcrossed mice treated in utero with MC developed tumors 12 months after birth. Our previous studies demonstrated that the differential susceptibility to tumor formation was not due to strain-specific differences in induction of phase I enzymes or in the formation or repair of DNA adducts [21]. The results presented in this study further suggest that strain-specific differences in phase II metabolism also cannot account for the differential susceptibility to transplacental tumorigenesis. It should be pointed out that we did not observe any sex-related differences in tumor incidence following in utero exposure to MC in any of our previous studies [15,17,36], and we pooled litters and did not separate the fetuses on the basis of sex in the results reported here. Thus, we cannot rule out sex-related differences in the levels of induction of the different GST enzymes during late gestation. Among the limitations of the current study include interpretation of some of the nonsignificant effects on GSTs. Here, levels in the liver for GST isoforms π and θ exhibited larger than expected variability, even after log-transformation to preserve normality and stabilize variability, which facilitated an effects size smaller than desired. This scenario was also observed for interaction effect in the ANOVA models for the 2-OH and 1-one metabolites. Here, a significant interaction effect was found for 2-OH ($P = 0.03$) but not for 1-one ($P = 0.87$). These two metabolites also exhibited larger than expected variability after being log-transformed, which facilitated smaller effects size. So while 2-OH was significant, caution in the interpretation of the interaction effect for 1-one is given. Similar attention is given for the main effects of treatment for 1-one ($P = 0.07$) and strain for 11,12-dione ($P = 0.47$).

It appears that, while metabolic activation of chemical carcinogens and the subsequent formation of mutagenic DNA adducts are necessary pre-requisites for tumor initiation, it is apparent that other downstream events play a role in ultimately determining the carcinogenic effects and individual response to DNA damaging agents. Identification of these novel factors will be important for understanding the molecular pathogenesis of cancer. These results and our recent studies [21,36] showing the relative resistance of B6 mice to tumor induction suggest the presence of a previously unidentified genetic locus that might influence susceptibility to cancer causing agents specifically following in utero exposure. The generally low levels of GSTs found during the fetal period thus has potential clinical implications. These results suggest that the fetus will be less able to detoxify exogenous agents than the adult organism, and thus may be sensitive to the potentially toxic effects of a variety of drugs and chemicals that would have no effect on the mother or adult organism. Identifying highly susceptible populations – such as the developing organism, pregnant women, and young children – that exhibit heightened sensitivity to carcinogenic agents should be a consideration in both the regulation of chemicals and drugs, as well as in the potential design of chemopreventive agents specifically developed to protect the fetus from environmental toxicants.

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REFERENCES

[1] Anderson LM, Diwan BA, Fear NT, Roman E. Critical windows of exposure for children's health: cancer in human epidemiological studies and neoplasms in experimental animal models. Environ Health Perspect 2000;108(Suppl. 3):573–94.
[2] Birnbaum LS, Fenton SE. Cancer and developmental exposure to endocrine disruptors. Environ Health Perspect 2003;111:389–94.
[3] Ma X, Buffler PA, Gunier RB, Dahl G, Smith MT, Reinier K, et al. Critical windows of exposure to household pesticides and risk of childhood leukemia. Environ Health Perspect 2002;110:955–60.
[4] Miller MS. Transplacental lung carcinogenesis: a pharmacogenetic mouse model for the modulatory role of cytochrome P450 1A1 on lung cancer initiation. Chem Res Toxicol 1994;7:471–81.
[5] Miller MS. Transplacental lung carcinogenesis: molecular mechanisms and pathogenesis. Toxicol Appl Pharmacol 2004;198:95–110.
[6] Rice JM. Perinatal period and pregnancy: intervals of high risk for chemical carcinogens. Environ Health Perspect 1979;29:23–7.
[7] Anderson LM, Jones AB, Miller MS, Chauhan DP. Metabolism of transplacental carcinogens. In: Napalkov NP, Rice JM, Tomatis L, Yamasaki H, editors. Transplacental and multigeneration carcinogenesis. Lyon, France: IARC Scientific Publications; 1989. p. 155–88.
[8] Hines RN, McCarver DG. The ontogeny of human drug-metabolizing enzymes: phase I oxidative enzymes. J Pharmacol Exp Ther 2002;300:355–60.
[9] Miller MS, Juchau MR, Guengerich FP, Nebert DW, Raucy JL. Drug metabolic enzymes in developmental toxicology. Fund Appl Toxicol 1996;34:165–75.
[10] Miller MS, McCarver DG, Bell DA, Eaton DL, Goldstein JA. Genetic polymorphisms in human drug metabolic enzymes. Fund Appl Toxicol 1997;40:1–14.

[11] Dutton GJ. Developmental aspects of drug conjugation, with special reference to glucuronidation. Annu Rev Pharmacol Toxicol 1978;18:17–35.
[12] McCarver DG, Hines RN. The ontogeny of human drug-metabolizing enzymes: phase II conjugation enzymes and regulatory mechanisms. J Pharmacol Exp Ther 2002;300:361–6.
[13] Miller MS, Jones AB, Park SS, Anderson LM. The formation of 3-methylcholanthrene-initiated lung tumors correlates with induction of cytochrome P450IA1 by the carcinogen in fetal but not adult mice. Toxicol Appl Pharmacol 1990;104:235–45.
[14] Anderson LM, Jones AB, Riggs CW, Ohshima M. Fetal mouse susceptibility to transplacental lung and liver carcinogenesis by 3-methylcholanthrene: positive correlation with responsiveness to inducers of aromatic hydrocarbon metabolism. Carcinogenesis 1985;6:1389–93.
[15] Wessner LL, Fan M, Schaeffer DO, McEntee MF, Miller MS. Mouse lung tumors exhibit specific Ki-ras mutations following transplacental exposure to 3-methylcholanthrene. Carcinogenesis 1996;17:1519–26.
[16] George JD, Manson JM. Strain-dependent differences in the metabolism of 3-methylcholanthrene by maternal, placental, and fetal tissues of C57BL/6J and DBA/2J mice. Cancer Res 1986;46:5671–5.
[17] Gressani KM, Leone-Kabler SL, O'Sullivan MG, Case LD, Malkinson AM, Miller MS. Strain-specific lung tumor formation in mice transplacentally exposed to 3-methylcholanthrene and post-natally exposed to butylated hydroxytoluene. Carcinogenesis 1999;20:2159–65.
[18] Miller MS, Leone-Kabler SL, Rollins LA, Wessner LL, Fan M, Schaeffer DO, et al. Molecular pathogenesis of transplacentally-induced mouse lung tumors. Exp Lung Res 1998;24:557–77.
[19] Leone-Kabler SL, Wessner LL, McEntee MF, D'Agostino RB, Miller MS. Ki-*ras* mutations are an early event and correlate with tumor stage in transplacentally induced murine lung tumors. Carcinogenesis 1997;18:1163–8.
[20] Miller MS, Gressani KM, Leone-Kabler S, Townsend AJ, Malkinson AM, O'Sullivan MG. Differential sensitivity to lung tumorigenesis following transplacental exposure of mice to polycyclic hydrocarbons, heterocyclic amines, and lung tumor promoters. Exp Lung Res 2000;26:709–30.
[21] Xu M, Nelson GB, Moore JE, McCoy TP, Dai J, Manderville RA, et al. Induction of Cyp1a1 and Cyp1b1 and formation of DNA adducts in C57BL/6, Balb/c, and F1 mice following in utero exposure to 3-methylcholanthrene. Toxicol Appl Pharmacol 2005;209:28–38.
[22] Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol 1995;30:445–600.
[23] Eaton DL, Bammler TK. Concise review of the glutathione S-transferases and their significance to toxicology. Toxicol Sci 1999;49:156–64.
[24] Landi S. Mammalian class theta GST and differential susceptibility to carcinogens: a review. Mutat Res 2000;463:247–83.
[25] Di Ilio C, Tiboni GM, Sacchetta P, Angelucci S, Bucciarelli T, Bellati U, et al. Time-dependent and tissue-specific variations of glutathione transferase activity during gestation in the mouse. Mech Ageing Dev 1995;78:47–62.
[26] Gressani KM, Leone-Kabler S, O'Sullivan MG, Townsend AJ, Miller MS. Prenatal toxicity and lack of carcinogenicity of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) following transplacental exposure. Toxicol Sci 2000;55:407–14.
[27] Hales BF, Huang C. Regulation of the Yp subunit of glutathione S-transferase P in rat embryos and yolk sacs during organogenesis. Biochem Pharmacol 1994;47:2029–37.
[28] Gallagher EP, Gardner JL. Comparative expression of two alpha class glutathione S-transferases in human adult and prenatal liver tissues. Biochem Pharmacol 2002;63:2025–36.
[29] Sims P. The metabolism of 3-methylcholanthrene and some related compounds by rat-liver homogenates. Biochem J 1966;98:215–28.
[30] Fields WR, Li Y, Townsend AJ. Protection by transfected glutathione S-transferase isozymes against carcinogen-induced alkylation of cellular macromolecules in human MCF-7 cells. Carcinogenesis 1994;15:1155–60.
[31] Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 1974;249:7130–9.
[32] Townsend AJ, Tu CP, Cowan KH. Expression of human mu or alpha class glutathione S-transferases in stably transfected human MCF-7 breast cancer cells: effect on cellular sensitivity to cytotoxic agents. Mol Pharmacol 1992;41:230–6.
[33] Townsend AJ, Fields WR, Haynes RL, Doss AJ, Li Y, Doehmer J, et al. Chemoprotective functions of glutathione S-transferases in cell lines induced to express specific isozymes by stable transfection. Chem Biol Interact 1998;111–112:389–407.
[34] Holm S. A simple sequentially rejective, multiple test procedure. Scand J Stat 1979;6:65–70.
[35] Westfall PH, Tobias RD, Rom D, Wolfinger RD. Multiple comparisons and multiple tests using SAS SAS Publishing; 1999.
[36] Jennings-Gee JE, Moore JE, Xu M, Dance ST, Kock ND, McCoy TP, et al. Strain specific induction of murine lung tumors following in utero exposure to 3-methylcholanthrene. Mol Carcinogen; In Press.
[37] Miller MS, Jones AB, Chauhan DP, Park SS, Anderson LM. Differential induction of fetal mouse liver and lung cytochromes P-450 by β-naphthoflavone and 3-methylcholanthrene. Carcinogenesis 1989;10:875–91.
[38] McQueen CA, Mitchell MK, Dang LN, Chau B, Tjalkens RB, Philbert MA. Prenatal expression of *N*-acetyltransferases in C57Bl/6 mice. Chem Biol Interact 2003;145:77–87.