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Benzo[a]pyrene effects on glycine N-methyltransferase mRNA expression and enzyme activity in *Fundulus heteroclitus* embryos

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Abstract

Benzo[a]pyrene (BaP) is a ubiquitous environmental polycyclic aromatic hydrocarbon (PAH) contaminant that is both a carcinogen and a developmental toxicant. We hypothesize that some of BaP's developmental toxicity may be mediated by effects on glycine N-methyltransferase (GNMT). GNMT is a mediator in the methionine and folate cycles, and the homotetrameric form enzymatically transfers a methyl group from S-adenosylmethionine (SAM) to glycine forming S-adenosylhomocysteine (SAH) and sarcosine. SAM homeostasis, as regulated by GNMT, is critically involved in regulation of DNA methylation, and altered GNMT expression is associated with liver pathologies. The homodimeric form of GNMT has been suggested as the 4S PAH-binding protein. To further study BaP-GNMT interactions, *Fundulus heteroclitus* embryos were exposed to waterborne BaP at 10 and 100 µg/L and both GNMT mRNA expression and enzyme activity were determined. Whole mount *in situ* hybridization showed GNMT mRNA expression was increased by BaP in the liver region of 7, 10 and 14 dpf *F. heteroclitus* embryos. In contrast to mRNA induction, *in vivo* BaP exposure decreased GNMT enzyme activity in 4, 10 and 14 dpf embryos. However, *in vitro* incubations of adult *F. heteroclitus* liver cytosol with BaP did not cause decreased enzyme activity. In conclusion, BaP exposure altered GNMT expression, which may represent a new target pathway for BaP-mediated embryonic toxicities and DNA methylation changes.

Keywords

Benzo[a]pyrene; Glycine N-methyltransferase; *Fundulus heteroclitus*.

1. Introduction

Benzo[a]pyrene (BaP), a model polycyclic aromatic hydrocarbon (PAH), is a known animal carcinogen and a probable human carcinogen by IARC's classification. The most widely recognized toxicity associated with PAHs is their mutagenicity and carcinogenicity in mammals, including humans (Mastrangelo et al. 1996) though their embryo and developmental toxicities are becoming more recognized (Billiard et al. 2008; Chen et al. 2005; Pershagen 1989; Tang et al. 2006; Tang et al. 2008)

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In the cytoplasm, BaP binds to both 8S and 4S PAH binding proteins (Raha *et al.* 1990). The 8S PAH binding protein is more commonly known as the aryl hydrocarbon receptor (AhR). The BaP-AhR complex translocates to the nucleus, heterodimerizes with ARNT and then transactivates genes, including CYP1A1, CYP1A2, CYP1B1 and some Phase II enzymes. BaP is bio-activated by the CYP1 family of P450 monooxygenases and/or other enzymes such as prostaglandin H synthase to the toxic metabolite BaP-7, 8-dihydrodiol 9, 10-epoxide (BPDE), which targets the genomic DNA by forming adducts. Besides genotoxic effects, BaP disrupts DNA methylation and thus also has epigenetic mechanisms of toxicity. The binding of BPDE to DNA affects the activities of DNA methyltransferase (Subach *et al.* 2006), and BaP causes sequence specific hypo- and hypermethylation in multiple breast cancer cell lines (Sadikovic *et al.* 2004). Currently, the identity and function of the 4S PAH binding protein is still under debate (Ogawa *et al.* 1997), however, it has been suggested to be glycine N-methyltransferase (GNMT; EC2.1.1.20) (Raha *et al.* 1994).

GNMT is the enzyme responsible for the transfer of a methyl group from S-adenosylmethionine (SAM) to glycine forming S-adenosylhomocysteine (SAH) and sarcosine, preserving the ratio of SAM/SAH, which is a sensitive indicator of the methylation capacity. GNMT makes up 1% or more of cytosolic proteins in the liver (Luka *et al.* 2009) and is a critical mediator in the methionine and folate cycles (Mato and Lu 2007). In mammals, the homotetrameric form of GNMT is enzymatically active and is the major folate binding protein in the liver (Cook and Wagner 1984). The homodimeric form has been suggested to be the 4S PAH binding protein (Bhat *et al.* 1997).

GNMT deficiency in humans is associated with various liver pathologies such as hypermethioninemia and hepatomegaly (Luka and Wagner 2003; Mudd *et al.* 2001). GNMT has been suggested to be a tumor suppressor gene and GNMT expression was down-regulated in hepatocellular carcinoma (HCC) (Tseng *et al.* 2003), cholangiocarcinoma (Huang *et al.* 2008) and prostate cancer tissues (Huang *et al.* 2007). Additionally, the higher expression of GNMT in females compared to males is proposed to be a possible mechanism explaining the much lower incidence of HCC in females than males (Liao *et al.* 2009). GNMT knockout mice spontaneously develop chronic hepatitis, glycogen storage disease (Liu *et al.* 2007), steatosis and HCC (Martinez-Chantar *et al.* 2008).

Although the controversy of GNMT and 4S PAH binding protein is not resolved, recent studies continue to find interactions between GNMT and BaP. Docking studies suggested a preference for BaP binding to the dimeric form of GNMT (Chen *et al.* 2004). In the same study, Chen *et al.* found that BaP exposure of GNMT transfected cells had decreased BPDE adduct formation and CYP1A1 enzyme activity. GNMT may sequester BaP, in turn, diminishing BaP's effects on the liver detoxification pathways and preventing cytotoxicity in HepG2 cells (Lee *et al.* 2006b). Recently, a functional xenobiotic response element (XRE) was identified in the 5' regulatory region of human GNMT and was inducible by BaP treatment *in vitro* (Lee *et al.* 2009). Therefore, it was suggested that GNMT activation may be involved in cellular defense mechanisms following BaP exposure.

Waterborne PAH exposure is capable of producing embryo toxicities and HCC in *F. heteroclitus* (Wang *et al.* submitted), a teleost that is exposed to environmental contaminants including PAHs and a highly recognized environmental toxicological model organism (Burnett *et al.* 2007). We have previously reported cloning of *F. heteroclitus* GNMT cDNA and the mRNA expression profile during embryogenesis measured by quantitative real-time PCR. GNMT mRNA was inducible in *F. heteroclitus* embryos by BaP exposure *in vivo* (Fang *et al.* 2009). In this study, we further characterized the cell/tissue-specific expression of GNMT mRNA in *F. heteroclitus* developing oocytes and embryos and then further investigated the BaP effects on embryonic GNMT mRNA expression and enzyme activity.

2. Methods

2.1 Fish source, care and handling

A parental population of *F. heteroclitus* was collected from an uncontaminated site at the Newport River near Beaufort Inlet, NC, USA and raised under the University Institutional Animal Care and Use Committee approved conditions. Sexually mature fish were bred and kept in salt water (20–25 parts per thousand (ppt), Instant Ocean, Cincinnati, OH). The fish were maintained at 20–25°C, 14:10 light-dark cycle. Adult fish were fed twice daily with tropical flake fish food (Tetramin, Tetra Werke, Germany) and live brine shrimp (Salt Creek, Inc., Salt Lake City, UT). First generation offspring, from wild parents, were used for the studies described here.

2.2 Embryo BaP exposure

Eggs and sperm were stripped from the parental *F. heteroclitus* population and mixed together for *in vitro* fertilization. Fertilized eggs were randomly sorted into three treatment groups, namely control dimethylsulfoxide (DMSO, 1 µl/mL), 10 and 100 µg/L BaP (Sigma-Aldrich, St. Louis, MO, stock solution 0.1 mg/mL in DMSO; final DMSO concentration was 1 µl/mL in all treatment groups). The pure water solubility of BaP ranges between 2.3 (Agency for Toxic Substances and Disease Registry (ATSDR) 1995) and 4 µg/L (Mackay and Shiu 1977). BaP is highly lipophilic with an octanol-water partition coefficient (K_{ow}) of 2.2×10^6 (Chiao et al. 1995). Therefore, the higher concentrations of BaP exposure could be considered to mimic either the maternal transfer and/or bioaccumulation of organic chemical to the lipid-rich ova (Kadokami et al. 2004). Ten eggs were pooled randomly and raised in 10 ml of salt water (~21 ppt). Exposures for each experimental sample began at approximately 4.5 hour post fertilization (hpf). Eggs were inspected daily for normal development. Water was changed and eggs were re-dosed every other day. For embryos prepared for *in situ* hybridization (ISH), 1-phenyl-2-thiourea (PTU, 0.003%, Sigma-Aldrich) was added to the culture water from 5 dpf until the end of the exposure to lessen pigmentation. Ten embryos per pool per time point were collected at 4, 7, 10 and 14 days post fertilization (dpf) (embryos would typically hatch at ~17 dpf). The 4 dpf, *F. heteroclitus* embryo is under active organodifferentiation, with defined heart chambers and a developing foregut. By 7 dpf, gut and liver are visible and the bilobed urinary bladder is formed. At 10 dpf, the mouth can open, and by 14 dpf, the swimming bladder has inflated (Armstrong and Child 1965).

For ISH, *F. heteroclitus* embryos were fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.4) overnight and stored at –20°C freezer after removal of the chorion membrane by watchmaker's forceps. For GNMT enzyme activity assays, embryos were collected and frozen at –80°C in 1 ml HEGD buffer (HEPES 10 mM, EDTA 1.5 mM, glycerol 10% v/v, DTT 1 mM, PMSF 0.5 mM and pH 8.0) until further analysis.

2.3 Whole mount *in situ* hybridization

Whole mount ISH was carried out as described elsewhere (Dong et al. 2002). The fragment of GNMT cDNA (Genbank Accession # FJ607947) used as template for RNA probe synthesis was generated by PCR amplification from *F. heteroclitus* liver cDNA, using primers: forward: 5'-CTGAAGTACGCGCTGAAG-3'; reverse: 5'-CAAAGGAGACCAGAGCGA-3'. The cDNA fragment contained 188 bp, from 277 to 464 bp (counted from translation start site) in the *F. heteroclitus* GNMT cDNA sequence, and was highly specific for GNMT according to a BLAST homology search. The fragment spanned across the putative GNMT exons 2, 3 and 4 to avoid genomic DNA binding (exon borders were determined by comparing splicing sites among *F. heteroclitus*, *Danio rerio* (#NM_212816.1) and *Homo sapiens* (#NM_01896) GNMT sequences). The PCR product was purified and inserted to pGEM-T® Easy Vector (Promega, Madison, MI). Sense and antisense digoxigenin ssRNA probes were synthesized by *in vitro*

transcription using T7 or SP6 RNA polymerase on GNMT plasmid linearized with *SpeI* or *ApaI* (New England Biolabs, Ipswich, MA). Following hybridization with probes overnight at 64°C, embryos were washed with 2x saline sodium citrate buffer (SSC, 0.15 M sodium chloride and 15 mM trisodium citrate, pH 7.0) and 0.2x SSC twice for 30 min, respectively. After blocking with 2% blocking reagent (Roche, Indianapolis, IN), embryos were incubated overnight with 4000x diluted anti-DIG antibody conjugated with alkaline phosphatase (Roche) at 4°C. The color reaction was carried out by incubation with BM-purple substrate (Roche). Yolks were carefully removed by watchmaker's forceps. GNMT expression was recorded with a digital camera (Optronics, Muskogee, OK) and dissecting microscope (Carl Zeiss, Thornwood, NY). Stained regions were outlined as regions of interest (ROIs) and the mean intensity was calculated (=sum intensity divided by ROI pixel area), with the use of KODAK 1D Image Analysis Software. For 7 and 14 dpf ISH, 9–17 embryos were collected for each treatment group; for 10 dpf ISH, 21–28 embryos were collected per group.

2.4 Paraffin embedding, sectioning and H&E staining

Adult *F. heteroclitus* ovary and 14 dpf BaP-exposed *F. heteroclitus* embryos were fixed in 4% (w/v) PFA in PBS (pH 7.4) overnight, followed by dehydration in increasing gradients of ethanol (70%, 80%, 90%, 95%, and 100% twice). After cleared in Clearify™ (American Master Tech Scientific, Lodi, CA), tissues were embedded in molten paraffin (Paraplast embedding media paraplast X-tra, Sigma). Sections of 5 µm thickness were prepared using a microtome (OlympusCut4055, Olympus American, San Jose, CA). For ovary, three adjacent sections were placed on three different slides with an “A” set for hematoxylin and eosin (H&E) staining, a “B” set for GNMT sense section ISH and a “C” set for GNMT anti-sense probe section ISH. For whole mount ISH processed embryos, two adjacent sections were placed on two different slides with an “A” set for eosin contrast staining, a “B” set for observation of the GNMT whole mount ISH signals only.

The sections were cleared with Clearify™ and a gradient of ethanol (100–70%). The slides were treated as indicated above and covered with cover-slips and observed under a digital camera and light microscope (Olympus America, Center Valley, PA).

2.5 Section *in situ* hybridization

Section ISH was carried out as described elsewhere (Dong and Willett 2008). The ovary sections were cleared in gradient of ethanol (100–70%) and hybridized with the sense and antisense *F. heteroclitus* GNMT probes as described above. Following hybridization overnight at 50°C, sections were washed with 2x SSC and 0.2x SSC twice for 20 min, respectively. After blocking with 2% blocking reagent (Roche), embryos were incubated overnight with 4000x diluted anti-DIG antibody conjugated with alkaline phosphatase (Roche) at 4°C. The color reaction was carried out by incubation with BM-purple substrate (Roche). The slides were covered with cover-slips and observed under a digital camera and light microscope (Olympus America, Center Valley, PA).

2.6 GNMT enzyme activity assay

Embryos (10 eggs/pool) or adult livers were homogenized in HEGD buffer. Homogenates were centrifuged at 10,000 × g for 1 hr and the resulted supernatants were the embryo or liver cytosolic preparations. Adult liver cytosol was prepared for method optimization and *F. heteroclitus* GNMT enzyme kinetics studies. Protein concentrations were measured by Bradford using BSA as a standard. The GNMT enzymatic activity was determined using the method of Cook and Wagner (Cook and Wagner 1984) with some modifications, and performed in duplicate. The GNMT assay mixture (100 µl) contained 0.1 M Tris HCl (pH 7.4), 5 mM DTT, 10 mM glycine (Fisher Scientific, Pittsburgh, PA), 1 mM S-adenosyl-L-[methyl-³H] methionine (0.02 µCi/reaction, PerkinElmer Life Sciences, Waltham, MA) + cold

SAM (Sigma-Aldrich), and 180–250 µg sample protein and was incubated at 25°C for 30–60 min. The reaction was terminated with 50 µl of 5% trichloroacetic acid, and 250 µl charcoal (38 mg/ml in 0.1 M acetic acid) was added to absorb the unreacted SAM. The tubes were then incubated at 4°C for 15 min and centrifuged for 3 min (14,000 × g). Supernatant (200 µl) was added to 5 ml of aqueous counting scintillant (Research Products International Corp., Mt. Prospect, IL) and assayed in a Packard Liquid Scintillation Analyzer (GMI, Inc., Ramsey, MN). Assay mixtures without protein served as negative controls and the values obtained were subtracted to give net counts. For method optimization, incubation time was set from 15–90 min and protein amount was loaded from 50–600 µg. To study enzyme kinetic parameters (V_{max} , K_m), 10 mM glycine was used while SAM concentrations varied from 0.02–3 mM or 1 mM SAM was used while glycine concentrations varied from 0.5–20 mM.

2.7 *In vitro* BaP effects on GNMT enzyme activity

Samples of adult liver cytosol were incubated with BaP to determine BaP's ability to inhibit GNMT *in vitro*. The protein samples (250 µg) were mixed with 1 µL of 1 mM, 5 mM or 10 mM BaP (in DMSO, final concentrations were 10 µM, 50 µM and 100 µM, respectively), 1 µL DMSO or water and incubated at 4°C for 1 hr prior to performing the GNMT activity assay as described above except pH was 9.0. Each treatment group had five samples.

2.8 Statistics

Results of ISH and embryonic enzyme activity were analyzed by GraphPad Prism 5.0 (La Jolla, CA) and presented as mean ± S.E. Statistical differences between treatment groups were determined using a one-way ANOVA followed by Neuman–Keulls post hoc test ($p < 0.05$). Data of the enzyme kinetic study was analyzed using nonlinear regression analyses (Michaelis-Menten equation).

3. Results

3.1 Expression of GNMT mRNA in *F. heteroclitus* oocytes

Developing oocytes were divided into stage I (previtellogenic up to 0.6 mm diameter), stage II (vitellogenesis 0.6–1.3 mm), stage III (maturation 1.3–1.9 mm with slight protein accumulation) and stage IV (maturation without protein accumulation) as defined by (Wallace and Selman 1985). We have further divided the stage I follicles into three size based categories: early stage I (stage Ia, 0–0.15 mm), mid stage I (stage Ib, 0.15–0.25 mm), and late stage I (stage Ic, 0.25–0.6 mm). Strongest expression of GNMT mRNA was detected in the ooplasm of stage Ia oocytes (Figure 1C and F). The signal gradually declined in the ooplasm of stage Ib and Ic oocytes (Figure 1I and L) and became barely detectable in later stages (data not shown).

3.2 Effects of BaP on GNMT mRNA expression in *F. heteroclitus* embryos

At 7 dpf, strong GNMT expression was detected in the liver of *F. heteroclitus* embryos by whole mount ISH (Figure 2). In addition to liver, GNMT was also expressed in the urinary bladder in 10 and 14 dpf embryos. The staining intensity in the liver area was quantitated, and BaP increased GNMT mRNA transcription in the liver of 7, 10 and 14 dpf embryos (Figure 3). Compared to controls, BaP at 10 and 100 µg/L induced liver GNMT mRNA expression by 20% and 43% in 7 dpf *F. heteroclitus* embryos, and by 10% and 9% in 10 dpf embryos, and by 20% and 32% in 14 dpf embryos. Paraffin sectioning more clearly showed the distribution of GNMT expression in liver and urinary bladder in 14 dpf BaP-exposed embryos (Figure 4).

3.3 *F. heteroclitus* GNMT enzyme kinetics

F. heteroclitus liver cytosol displayed Michaelis-Menten kinetics for both S-adenosylmethionine (SAM) and glycine substrates when one of the substrates was present in

limited concentrations and the other substrate was in excess. The extrapolated K_m values for SAM and glycine were 0.18 ± 0.03 and 3.24 ± 0.53 mM, respectively (Figure 5). Estimated V_{max} for SAM and glycine were 18.80 ± 0.78 and 22.29 ± 1.17 pmol/mg*min, respectively. As indicated in Figure 5, the curves reached plateau activities with 1 mM SAM and 10 mM glycine, suggesting the saturation concentrations for the enzyme. Therefore, these concentrations were selected for GNMT activity analyses. GNMT activities were linear with reaction time (15 – 90 min) and protein amount (50 – 600 μ g) (data not shown).

3.4 BaP-mediated inhibition of GNMT enzyme activity

GNMT enzyme activity exists in unfertilized eggs. Constitutive GNMT activity was significantly higher (about two fold) in 4 dpf embryos compared to all other time points tested (Figure 6). Waterborne BaP exposure decreased GNMT enzyme activity in 4, 10 and 14 dpf embryos. BaP exposure of 10 and 100 μ g/L inhibited embryonic GNMT enzyme activity by 33% and 36% in 4 dpf *F. heteroclitus* embryos, by 33% and 55% in 10 dpf embryos, and by 3% and 23% in 14 dpf embryos. However, *in vitro* incubation with BaP (10–100 μ M) had no effect on constitutive GNMT enzyme activity (Figure 7).

4. Discussion

F. heteroclitus is a estuarine teleost found in contaminated environments along the Atlantic coast of the US with relevant human diseases including cancer and reproductive/developmental deficits (Meyer et al. 2002; Pait 2001; Vogelbein et al. 1990). Because of its small size, external development, and transparent chorion, it is suitable to study developmental toxicities. The *F. heteroclitus* GNMT deduced protein has 295 amino acids and 74% identity with human GNMT (Fang et al. 2009). As shown in Table 1, the *F. heteroclitus* liver GNMT K_m s compared similarly with previously reported mammalian kinetic values. Therefore, *F. heteroclitus* is a relevant and useful model organism to further investigate GNMT functions in embryogenesis and BaP-mediated developmental toxicity.

GNMT protein was first discovered in guinea pig livers (Blumenstein and Williams 1963) and then purified ten years later (Heady and Kerr 1973). Sequences for GNMT have been identified in many other species including human, mouse, rat, rabbit, cow, dog, chicken, zebrafish, medaka and fruitfly, etc. In mammals, GNMT mRNA and protein expression is highest in the liver, pancreas, prostate and kidney (proximal kidney tubules) (Yeo and Wagner 1994). But to date, GNMT expression during oogenesis, embryogenesis and its developmental significance have not been reported in any other organisms. In this study, we found that GNMT mRNA exists in developing oocytes, was expressed throughout development, and was mainly localized in the *F. heteroclitus* embryonic liver and urinary bladder. GNMT enzyme activity was also detected in unfertilized eggs and developing embryos suggesting its critical role during early development.

In our previous mRNA measurements using quantitative RT-PCR, we found unfertilized *F. heteroclitus* eggs had the highest expression of GNMT mRNA, about 19-fold higher than 4 dpf embryo expression (Fang et al. 2009), and hypothesized GNMT was possibly a maternally deposited mRNA. In this study, we confirmed that GNMT was actively transcribed and accumulated in growing oocytes and detected GNMT activities in unfertilized eggs. However, the GNMT activity did not peak in unfertilized eggs (as could be predicted based on mRNA levels) but rather later during development. This suggests a large portion of the maternally deposited GNMT mRNA was not translated to GNMT protein before fertilization. Generally, as discussed in (Alizadeh et al. 2005), during meiotic maturation and in embryos shortly after fertilization accumulated maternal mRNA species degrade rapidly for protein synthesis if they encode essential proteins for these specific events. This could account for our previous results that constitutive whole embryo GNMT mRNA expression dropped dramatically post

fertilization. In 2 and 3 dpf *F. heteroclitus* embryos, GNMT expression was still significantly higher than when expression leveled off between 4 and 14 dpf. Possibly resulting from the rapid translation of maternally deposited mRNA, GNMT activity was also significantly higher in early development (4 dpf) than later time-points, suggesting GNMT activity is critical to *F. heteroclitus* embryogenesis at early stages corresponding to periods of rapid organogenesis (Armstrong and Child 1965). Interestingly, the temporal expression pattern of embryonic GNMT is very similar to DNA methyltransferase 1 (DNMT1). Maternal transcripts of the zebrafish DNMT1 are present at high levels in early embryos and then the mRNA abundance decreases after the blastula stage (Martin et al. 1999; Mhanni and McGowan 2002).

In this study, we found by ISH that the developmental toxicant, BaP, induced GNMT mRNA expression modestly in the embryo liver region in 7, 10, 14 dpf. We also did ISH on 4 dpf embryos, but embryos are undergoing organogenesis and organs like liver and urinary bladder are not easily visualized. It was difficult to distinguish and quantitate the tissue specific expression by ISH at this time point. However, the same trends in GNMT mRNA induction by BaP were found using whole mount ISH and our earlier quantitative RT-PCR results on homogenized whole *F. heteroclitus* embryo pools where GNMT mRNA induction at 3, 10, and 14 dpf by BaP treatment was detected (Fang et al. 2009). While studies of BaP effects on GNMT are very limited, BaP exposure also induced GNMT mRNA expression in HepG2 cells *in vitro* (Lee et al. 2006a). Induction of GNMT expression is consistent with the recent recognition that the 5'-regulatory region of the human GNMT contains a BaP inducible XRE (Lee et al. 2009). While the *F. heteroclitus* GNMT promoter sequence data are not available, our bioinformatics analysis using the Transcription Element Search System (TESS, combined search) on the GNMT promoter of *Danio rerio* (zebrafish), a more genetically established teleost model, revealed five putative AhR or AhR/ARNT binding sites between -2000 nt and the transcription start site (Table 2). Collectively, these new data suggest that the AhR may have a role in GNMT expression and may explain the BaP-induced mRNA expression we found in *F. heteroclitus* embryos. The AhR-GNMT relationship is further suggested in AhR null mice where liver constitutive GNMT expression is significantly lower than in wild-type mice (Boutros et al. 2009). Therefore, the transcriptional induction by BaP was possibly mediated through the AhR and XRE in the upstream region of the GNMT gene.

In contrast to mRNA induction, BaP exposure significantly reduced GNMT enzyme activity. To determine if BaP is a direct inhibitor of GNMT, BaP was added to the enzyme reaction system, but it did not alter the GNMT enzyme activity in *F. heteroclitus* liver cytosol (Figure 7). Our results contrasted to a similar *in vitro* study (Chen et al. 2004) showing that when BaP was incorporated into a recombinant human GNMT assay system, GNMT activity was reduced by 50% when compared to control DMSO. In either case, the *in vitro* studies do not represent the *in vivo* system where BaP metabolites may also be acting as GNMT inhibitors.

Another possible mechanism to explain enzyme inhibition is that the BaP-mediated mRNA increase is reflected in more post translational assembly of the homodimeric BaP-binding form. As a result, the assembly of the enzymatically active homotetrameric form of GNMT could be decreased. In fact, Bhat and coworkers found that BaP altered the post-translational modification of GNMT and resulted in formation of GNMT homodimer, which is the PAH binding but nonenzymatic form (Bhat et al. 1997). In our case, BaP may shift the GNMT translation from tetramer to dimer and thus reduce the GNMT enzyme activity. To specifically address this latter hypothesis a fish-specific GNMT antibody to measure relative homodimer vs. tetramer protein abundances following BaP exposure is necessary. Unfortunately, the commercially available human GNMT antibody does not cross-react with the *F. heteroclitus* protein (data not shown).

To date, studies suggest GNMT is involved in cellular defense mechanisms in response to environmental stressors and disease states. After BaP exposure, GNMT transfected cells have reduced BPDE formation (Chen et al. 2004), and cell lines expressing constitutively higher GNMT expression have less cytotoxicity than low GNMT expressing cell lines (Lee et al. 2006). Carcinogenic effects of aflatoxin B1 were reduced in GNMT transgenic mice that express human GNMT in the liver and kidney (Yen et al. 2009). GNMT enzyme activity and protein expression were induced after exposure to various xenobiotics, such as ethanol (Villanueva and Halsted 2004), retinoid compounds (Rowling and Schalinske 2001), glucocorticoids (Rowling and Schalinske 2003), excess dietary methionine (Rowling et al. 2002a) and streptozotocin (used to establish diabetic animal models) (Nieman et al. 2006). Thus, GNMT induction is responsive to various environmental stressors and is possibly involved in protective mechanisms. On the other hand, diminished GNMT expression is commonly seen in various cancer states including HCC, prostate cancer and cholangiocarcinoma (Tseng et al. 2003; Huang et al. 2007; Huang et al. 2008). Clearly, there remains much to resolve with respect to the roles of GNMT in disease and the relative utility of message expression versus enzyme activity as a biomarker of effect.

Another concern about the diminished developmental GNMT activities *in vivo* after BaP exposure is the potential effect on SAM/SAH homeostasis and DNA methylation status. A number of more recent studies have established a relationship between altered GNMT expression and DNA methylation (Lu et al. 2000; Mato et al. 1997; Rowling et al. 2002b). Altered GNMT activity will change the ratio of SAM/SAH and affect the methylation capacity in the body. Compared to control wild-type animals GNMT knockout mice had 7-fold more methionine, 35-fold more SAM and a 100-fold increase in the SAM:SAH ratio (Luka et al. 2006) and changes in global or chromosome specific DNA methylation patterns (Martinez-Chantar et al. 2008). These studies highlight the ability for altered GNMT, SAM and SAH homeostasis to affect critical cellular pathways epigenetically. Our future studies will focus on measuring the embryonic SAM and SAH levels and subsequent global or gene specific methylation changes after BaP exposure in this model organism. If these changes are detected, it will expand our knowledge on the epigenetic mechanisms of BaP toxicity.

PAHs are ubiquitous environmental contaminants which cause various embryo/fetal toxicities and cancer in both fish (Incardona et al. 2004; Wassenberg and Di Giulio 2004) and humans (Choi et al. 2008; Siddiqui et al. 2008; Stjernfeldt et al. 1986). BaP exposure during embryo-fetal development decreases GNMT enzyme activity and may change the pattern of DNA methylation of critical genes at critical times in development, have life-long effects on gene expression and phenotype, then result in diseases later in life (Szyf 2009; Tremblay and Hamet 2008). Recent studies challenge the idea that DNA methylation is irreversible, and some success in epigenetic therapy has been reported (Delcuve et al. 2009). Therefore, better understanding of whether BaP targets methylation homeostasis pathways such as GNMT and epigenetic consequences of that interaction are critical to prevent or potentially treat PAH toxicity.

5. Conclusion

In this study, we found that BaP exposure tissue-specifically induced GNMT mRNA expression measured by whole mount ISH but decreased GNMT enzyme activity in *F. heteroclitus* embryos throughout development. BaP-mediated changes in GNMT may represent a novel mechanism involved in BaP-induced embryo toxicity and DNA methylation changes.

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Abbreviations

BaP	Benzo[a]pyrene
BPDE	BaP-7, 8-dihydrodiol 9, 10-epoxide
dpf	Days post fertilization
PTU	1-phenyl-2-thiourea
PAH	Polycyclic aromatic hydrocarbon
HCC	Hepatocellular carcinoma
hpf	Hours post fertilization
ISH	<i>In situ</i> hybridization
GNMT	Glycine N-methyltransferase
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
XRE	Xenobiotic response element

Reference List

- Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological profile for polycyclic aromatic hydrocarbons (PAHs). ATSDR; Atlanta, GA: 1995. p. 217
- Alizadeh Z, Kageyama S, Aoki F. Degradation of maternal mRNA in mouse embryos: selective degradation of specific mRNAs after fertilization. *Mol Reprod Dev* 2005;72:281–290. [PubMed: 16094646]
- Armstrong PB, Child JS. Stages in the normal development of *Fundulus heteroclitus*. *The Marine Biological Laboratory* 1965;128:143–168.
- Bhat R, Wagner C, Bresnick E. The homodimeric form of glycine N-methyltransferase acts as a polycyclic aromatic hydrocarbon-binding receptor. *Biochemistry* 1997;36:9906–9910. [PubMed: 9245423]
- Billiard SM, Meyer JN, Wassenberg DM, Hodson PV, Di Giulio RT. Nonadditive effects of PAHs on Early Vertebrate Development: mechanisms and implications for risk assessment. *Toxicol Sci* 2008;105:5–23. [PubMed: 18156145]
- Blumenstein J, Williams GR. Glycine methyltransferase. *Can J Biochem Physiol* 1963;41:201–210. [PubMed: 13971907]
- Boutros PC, Bielefeld KA, Pohjanvirta R, Harper PA. Dioxin-dependent and dioxin-independent gene batteries: comparison of liver and kidney in AHR-null mice. *Toxicol Sci* 2009;112:245–256. [PubMed: 19759094]
- Burnett KG, Bain LJ, Baldwin WS, Callard GV, Cohen S, Di Giulio RT, Evans DH, Gomez-Chiarri M, Hahn ME, Hoover CA, Karchner SI, Katoh F, Maclatchy DL, Marshall WS, Meyer JN, Nacci DE, Oleksiak MF, Rees BB, Singer TD, Stegeman JJ, Towle DW, Van Veld PA, Vogelbein WK, Whitehead A, Winn RN, Crawford DL. *Fundulus* as the Premier Teleost Model in Environmental Biology: Opportunities for New Insights Using Genomics. *Comp Biochem Physiol Part D Genomics Proteomics* 2007;2:257–286. [PubMed: 18071578]
- Chen C, Wang X, Wang L, Yang F, Tang G, Xing H, Ryan L, Lasley B, Overstreet JW, Stanford JB, Xu X. Effect of environmental tobacco smoke on levels of urinary hormone markers. *Environ Health Perspect* 2005;113:412–417. [PubMed: 15811831]

- Chen SY, Lin JR, Darbha R, Lin P, Liu TY, Chen YM. Glycine N-methyltransferase tumor susceptibility gene in the benzo(a)pyrene-detoxification pathway. *Cancer Res* 2004;64:3617–3623. [PubMed: 15150120]
- Chiao F, Currie R, McKone TE. Final draft report: intermedia transfer factors for contaminants found at hazardous waste sites. *Benzo(a)pyrene* 1995:2.
- Choi H, Rauh V, Garfinkel R, Tu Y, Perera FP. Prenatal exposure to airborne polycyclic aromatic hydrocarbons and risk of intrauterine growth restriction. *Environ Health Perspect* 2008;116:658–665. [PubMed: 18470316]
- Cook RJ, Wagner C. Glycine N-methyltransferase is a folate binding protein of rat liver cytosol. *Proc Natl Acad Sci USA* 1984;81:3631–3634. [PubMed: 6587377]
- Delcuve GP, Rastegar M, Davie JR. Epigenetic control. *J Cell Physiol* 2009;219:243–250. [PubMed: 19127539]
- Dong W, Teraoka H, Yamazaki K, Tsukiyama S, Imani S, Imagawa T, Stegeman JJ, Peterson RE, Hiraga T. 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in the zebrafish embryo: local circulation failure in the dorsal midbrain is associated with increased apoptosis. *Toxicol Sci* 2002;69:191–201. [PubMed: 12215674]
- Dong W, Willett KL. Local expression of CYP19A1 and CYP19A2 in developing and adult killifish (*Fundulus heteroclitus*). *Gen Comp Endocrinol* 2008;155:307–317. [PubMed: 17582409]
- Fang X, Dong W, Thornton C, Scheffler B, Willett KL. Benzo(a)pyrene induced glycine N-methyltransferase messenger RNA expression in *Fundulus heteroclitus* embryos. *Mar Environ Res*. 2009
- Heady JE, Kerr SJ. Purification and characterization of glycine N-methyltransferase. *J Biol Chem* 1973;248:69–72. [PubMed: 4692843]
- Huang YC, Chen M, Shyr YM, Su CH, Chen CK, Li AF, Ho DM, Chen YM. Glycine N-methyltransferase is a favorable prognostic marker for human cholangiocarcinoma. *J Gastroenterol Hepatol* 2008;23:1384–1389. [PubMed: 18624901]
- Huang YC, Lee CM, Chen M, Chung MY, Chang YH, Huang WJ, Ho DM, Pan CC, Wu TT, Yang S, Lin MW, Hsieh JT, Chen YM. Haplotypes, loss of heterozygosity, and expression levels of glycine N-methyltransferase in prostate cancer. *Clin Cancer Res* 2007;13:1412–1420. [PubMed: 17332283]
- Incardona JP, Collier TK, Scholz NL. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. *Toxicol Appl Pharmacol* 2004;196:191–205. [PubMed: 15081266]
- Kadokami K, Takeishi M, Kuramoto M, Ono Y. Maternal transfer of organochlorine pesticides, polychlorinated dibenzo-p-dioxins, dibenzofurans, and coplanar polychlorinated biphenyls in frogs to their eggs. *Chemosphere* 2004;57:383–389. [PubMed: 15331265]
- Kloor D, Karnahl K, Kompf J. Characterization of glycine N-methyltransferase from rabbit liver. *Biochem Cell Biol* 2004;82:369–374. [PubMed: 15181470]
- Lee CM, Chen SY, Lee YC, Huang CY, Chen YM. Benzo[a]pyrene and glycine N-methyltransferase interactions: gene expression profiles of the liver detoxification pathway. *Toxicol Appl Pharmacol* 2006a;214:126–135. [PubMed: 16545412]
- Lee CM, Chen SY, Lee YC, Huang CY, Chen YM. Benzo[a]pyrene and glycine N-methyltransferase interactions: gene expression profiles of the liver detoxification pathway. *Toxicol Appl Pharmacol* 2006b;214:126–135. [PubMed: 16545412]
- Lee CM, Shih YP, Wu CH, Chen YM. Characterization of the 5' regulatory region of the human Glycine N-methyltransferase gene. *Gene* 2009;443:151–157. [PubMed: 19439180]
- Liao YJ, Liu SP, Lee CM, Yen CH, Chuang PC, Chen CY, Tsai TF, Huang SF, Lee YH, Chen YM. Characterization of a glycine N-methyltransferase gene knockout mouse model for hepatocellular carcinoma: Implications of the gender disparity in liver cancer susceptibility. *Int J Cancer* 2009;124:816–826. [PubMed: 19035462]
- Liu SP, Li YS, Chen YJ, Chiang EP, Li AF, Lee YH, Tsai TF, Hsiao M, Huang SF, Chen YM. Glycine N-methyltransferase^{-/-} mice develop chronic hepatitis and glycogen storage disease in the liver. *Hepatology* 2007;46:1413–1425. [PubMed: 17937387]

- Lu SC, Huang ZZ, Yang H, Mato JM, Avila MA, Tsukamoto H. Changes in methionine adenosyltransferase and S-adenosylmethionine homeostasis in alcoholic rat liver. *Am J Physiol Gastrointest Liver Physiol* 2000;279:G178–G185. [PubMed: 10898761]
- Luka Z, Capdevila A, Mato JM, Wagner C. A glycine N-methyltransferase knockout mouse model for humans with deficiency of this enzyme. *Transgenic Res* 2006;15:393–397. [PubMed: 16779654]
- Luka Z, Mudd SH, Wagner C. Glycine N-methyltransferase and regulation of S-adenosylmethionine levels. *J Biol Chem*. 2009
- Luka Z, Wagner C. Effect of naturally occurring mutations in human glycine N-methyltransferase on activity and conformation. *Biochem Biophys Res Commun* 2003;312:1067–1072. [PubMed: 14651980]
- Mackay D, Shiu W. Aqueous solubility of polynuclear aromatic hydrocarbons. *J Chem Eng Data* 1977;22:399–402.
- Martin CC, Laforest L, Akimenko MA, Ekker M. A role for DNA methylation in gastrulation and somite patterning. *Dev Biol* 1999;206:189–205. [PubMed: 9986732]
- Martinez-Chantar ML, Vazquez-Chantada M, Ariz U, Martinez N, Varela M, Luka Z, Capdevila A, Rodriguez J, Aransay AM, Matthiesen R, Yang H, Calvisi DF, Esteller M, Fraga M, Lu SC, Wagner C, Mato JM. Loss of the glycine N-methyltransferase gene leads to steatosis and hepatocellular carcinoma in mice. *Hepatology* 2008;47:1191–1199. [PubMed: 18318442]
- Mastrangelo G, Fadda E, Marzia V. Polycyclic aromatic hydrocarbons and cancer in man. *Environ Health Perspect* 1996;104:1166–1170. [PubMed: 8959405]
- Mato JM, Alvarez L, Ortiz P, Pajares MA. S-adenosylmethionine synthesis: molecular mechanisms and clinical implications. *Pharmacol Ther* 1997;73:265–280. [PubMed: 9175157]
- Mato JM, Lu SC. Role of S-adenosyl-L-methionine in liver health and injury. *Hepatology* 2007;45:1306–1312. [PubMed: 17464973]
- Meyer JN, Nacci DE, Di Giulio RT. Cytochrome P4501A (CYP1A) in killifish (*Fundulus heteroclitus*): heritability of altered expression and relationship to survival in contaminated sediments. *Toxicol Sci* 2002;68:69–81. [PubMed: 12075112]
- Mhanni AA, McGowan RA. Variations in DNA (cytosine-5)-methyltransferase-1 expression during oogenesis and early development of the zebrafish. *Dev Genes Evol* 2002;212:530–533. [PubMed: 12459921]
- Mudd SH, Cerone R, Schiaffino MC, Fantasia AR, Minniti G, Caruso U, Lorini R, Watkins D, Matiaszuk N, Rosenblatt DS, Schwahn B, Rozen R, LeGros L, Kotb M, Capdevila A, Luka Z, Finkelstein JD, Tangerman A, Stabler SP, Allen RH, Wagner C. Glycine N-methyltransferase deficiency: a novel inborn error causing persistent isolated hypermethioninaemia. *J Inherit Metab Dis* 2001;24:448–464. [PubMed: 11596649]
- Nieman KM, Hartz CS, Szegedi SS, Garrow TA, Sparks JD, Schalinske KL. Folate status modulates the induction of hepatic glycine N-methyltransferase and homocysteine metabolism in diabetic rats. *Am J Physiol Endocrinol Metab* 2006;291:E1235–E1242. [PubMed: 16835399]
- Ogawa H, Gomi T, Fujioka M. Mammalian glycine N-methyltransferases. Comparative kinetic and structural properties of the enzymes from human, rat, rabbit and pig livers. *Comp Biochem Physiol B* 1993;106:601–611. [PubMed: 8281755]
- Ogawa H, Gomi T, Imamura T, Kobayashi M, Huh N. Rat liver 4S-benzo[a]pyrene-binding protein is distinct from glycine N-methyltransferase. *Biochem Biophys Res Commun* 1997;233:300–304. [PubMed: 9144528]
- Pait, AS. Reproductive endocrine disruption in the killifish *Fundulus heteroclitus* in the Chesapeake Bay. University of Maryland; College Park: 2001.
- Pakhomova S, Luka Z, Grohmann S, Wagner C, Newcomer ME. Glycine N-methyltransferases: a comparison of the crystal structures and kinetic properties of recombinant human, mouse and rat enzymes. *Proteins* 2004;57:331–337. [PubMed: 15340920]
- Pershagen G. Childhood cancer and malignancies other than lung cancer related to passive smoking. *Mutat Res* 1989;222:129–135. [PubMed: 2645517]
- Raha A, Reddy V, Houser W, Bresnick E. Binding characteristics of 4S PAH-binding protein and Ah receptor from rats and mice. *J Toxicol Environ Health* 1990;29:339–355. [PubMed: 2157855]

- Raha A, Wagner C, MacDonald RG, Bresnick E. Rat liver cytosolic 4 S polycyclic aromatic hydrocarbon-binding protein is glycine N-methyltransferase. *J Biol Chem* 1994;269:5750–5756. [PubMed: 8119914]
- Rowling MJ, McMullen MH, Chipman DC, Schalinske KL. Hepatic glycine N-methyltransferase is up-regulated by excess dietary methionine in rats. *J Nutr* 2002a;132:2545–2550. [PubMed: 12221207]
- Rowling MJ, McMullen MH, Schalinske KL. Vitamin A and its derivatives induce hepatic glycine N-methyltransferase and hypomethylation of DNA in rats. *J Nutr* 2002b;132:365–369. [PubMed: 11880556]
- Rowling MJ, Schalinske KL. Retinoid compounds activate and induce hepatic glycine N-methyltransferase in rats. *J Nutr* 2001;131:1914–1917. [PubMed: 11435507]
- Rowling MJ, Schalinske KL. Retinoic acid and glucocorticoid treatment induce hepatic glycine N-methyltransferase and lower plasma homocysteine concentrations in rats and rat hepatoma cells. *J Nutr* 2003;133:3392–3398. [PubMed: 14608049]
- Sadikovic B, Haines TR, Butcher DT, Rodenhiser DI. Chemically induced DNA hypomethylation in breast carcinoma cells detected by the amplification of intermethylated sites. *Breast Cancer Res* 2004;6:R329–R337. [PubMed: 15217500]
- Siddiqui AR, Gold EB, Yang X, Lee K, Brown KH, Bhutta ZA. Prenatal exposure to wood fuel smoke and low birth weight. *Environ Health Perspect* 2008;116:543–549. [PubMed: 18414641]
- Stjernfeldt M, Ludvigsson J, Berglund K, Lindsten J. Maternal smoking during pregnancy and the risk of childhood cancer. *Lancet* 1986;2:687–688. [PubMed: 2876156]
- Subach OM, Baskunov VB, Darii MV, Maltseva DV, Alexandrov DA, Kirsanova OV, Kolbanovskiy A, Kolbanovskiy M, Johnson F, Bonala R, Geacintov NE, Gromova ES. Impact of benzo[a]pyrene-2'-deoxyguanosine lesions on methylation of DNA by SssI and HhaI DNA methyltransferases. *Biochemistry* 2006;45:6142–6159. [PubMed: 16681387]
- Szyf M. The early life environment and the epigenome. *Biochim Biophys Acta*. 2009
- Tang D, Li TY, Liu JJ, Chen YH, Qu L, Perera F. PAH-DNA adducts in cord blood and fetal and child development in a Chinese cohort. *Environ Health Perspect* 2006;114:1297–1300. [PubMed: 16882543]
- Tang D, Li TY, Liu JJ, Zhou ZJ, Yuan T, Chen YH, Rauh VA, Xie J, Perera F. Effects of prenatal exposure to coal-burning pollutants on children's development in China. *Environ Health Perspect* 2008;116:674–679. [PubMed: 18470301]
- Tremblay J, Hamet P. Impact of genetic and epigenetic factors from early life to later disease. *Metabolism* 2008;57(Suppl 2):S27–S31. [PubMed: 18803962]
- Tseng TL, Shih YP, Huang YC, Wang CK, Chen PH, Chang JG, Yeh KT, Chen YM, Buetow KH. Genotypic and phenotypic characterization of a putative tumor susceptibility gene, GNMT, in liver cancer. *Cancer Res* 2003;63:647–654. [PubMed: 12566309]
- Villanueva JA, Halsted CH. Hepatic transmethylation reactions in micropigs with alcoholic liver disease. *Hepatology* 2004;39:1303–1310. [PubMed: 15122759]
- Vogelbein WK, Fournie JW, Van Veld PA, Huggett RJ. Hepatic neoplasms in the mummichog *Fundulus heteroclitus* from a creosote-contaminated site. *Cancer Res* 1990;50:5978–5986. [PubMed: 2168287]
- Wallace RA, Selman K. Major protein changes during vitellogenesis and maturation of *Fundulus* oocytes. *Dev Biol* 1985;110:492–498. [PubMed: 4018410]
- Wang L, Thornton C, Thornton MW, Camus A, Willett KL. Histologic and immunohistochemical characterization of CYP1C1 and CYP1A expression in PAH-induced. *Fundulus* hepatic lesions. submitted.
- Wassenberg DM, Di Giulio RT. Synergistic embryotoxicity of polycyclic aromatic hydrocarbon aryl hydrocarbon receptor agonists with cytochrome P4501A inhibitors in *Fundulus heteroclitus*. *Environ Health Perspect* 2004;112:1658–1664. [PubMed: 15579409]
- Yen CH, Hung JH, Ueng YF, Liu SP, Chen SY, Liu HH, Chou TY, Tsai TF, Darbha R, Hsieh LL, Chen YM. Glycine N-methyltransferase affects the metabolism of aflatoxin B1 and blocks its carcinogenic effect. *Toxicol Appl Pharmacol* 2009;235:296–304. [PubMed: 19146867]
- Yeo EJ, Wagner C. Tissue distribution of glycine N-methyltransferase, a major folate-binding protein of liver. *Proc Natl Acad Sci USA* 1994;91:210–214. [PubMed: 8278367]

Figure 1.

Expression of GNMT mRNA in adult oocytes. Adult ovaries were sectioned and visualized by H&E staining (A, D, G and J) or *in situ* hybridization using GNMT sense probe (B, E, H and K) or antisense probe (C, F, I and L). Strong expression of GNMT mRNA was detected in the ooplasm of stage Ia oocytes (primary growth stage, C and F). Signal gradually decreased and became barely detectable by stage Ib and Ic oocytes (previtellogenic follicle stage, I and L, respectively). Oocytes were characterized by the presence of yolk granules in ooplasm (Wallace and Selman 1985) and size.

Figure 2.

BaP induced GNMT mRNA expression in the liver of developing *F. heteroclitus* embryos measured by whole mount *in situ* hybridization. Embryos were exposed to DMSO, or BaP (10 or 100 µg/L, final DMSO concentration was 1 µl/mL in all treatment groups) from 4.5 hour post fertilization (hpf) to 7, 10 or 14 days post fertilization (dpf). From 5 dpf to the end of exposure, PTU was added to reduce pigmentation. Arrows (black) show GNMT expression signals (in purple color) in the livers of 7, 10 and 14 dpf *F. heteroclitus*. Arrow heads (red) show GNMT signals in the urinary bladders of 10 and 14 dpf *F. heteroclitus*. Panel A, E and I indicate nonspecific binding in control (DMSO treated) embryos by using GNMT sense probe. Chorion and yolk were removed from the embryo during the experimental procedure.

Figure 3.

Intensity of GNMT expression in the liver region of 7, 10 and 14 dpf *F. heteroclitus* embryos by whole-mount *in situ* hybridization (ISH). Expressed regions were analyzed by means of KODAK 1D Image Analysis Software by creating regions of interest. * Indicates significant increase of GNMT expression between the BaP treated and control groups (ANOVA, * $p < 0.05$, ** $p < 0.01$). For 7 and 14 dpf ISH, each bar represents mean $\pm$ SE of 9–17 embryos. For 10 dpf ISH, each bar represents mean $\pm$ SE of 21–28 embryos.

Figure 4.

Paraffin sections of 14 dpf BaP-exposed embryos stained by GNMT whole mount *in situ* hybridization (purple). Embryos were exposed to 100 μg/L BaP from 4.5 hpf to 14 days. A is the longitudinal section of the whole fish embryo; B is the eosin contrast staining (red) of the adjacent slide of A; C and D show strong GNMT signals in the liver and urinary bladder, respectively. E and F are magnification of the rectangular region of C and D, respectively.

Figure 5.

Determination of GNMT kinetics with *F. heteroclitus* liver cytosol. Liver cytosol (250 μ g) was used and reactions were performed at 25°C for 30 min. (A) K_m (SAM) was 0.18 ± 0.03 mM ($R^2 = 0.92$) and V_{max} was 18.80 ± 0.78 pmol/mg*min. Reactions were incubated with 10 mM glycine when the SAM concentration was varied from 0.02–3 mM. (B) K_m (glycine) was 3.24 ± 0.53 mM (R^2 is 0.91) and V_{max} was 22.29 ± 1.17 pmol/mg*min. Reactions were incubated with 1 mM SAM when the glycine concentration was varied from 0.5–20 mM.

Figure 6.

BaP waterborne exposure inhibited GNMT enzyme activity in *F. heteroclitus* embryos. Embryo cytosolic protein (180 µg) was used and reactions were performed with 1 mM SAM and 10 mM glycine at 25°C for 60 min. Each bar represents n = 5 pools, 10 embryos per pool, * p<0.05, ** p<0.01. Constitutive GNMT enzyme activity was significantly higher at 4 dpf (b).

Figure 7.

In vitro effects of BaP on adult *F. heteroclitus* liver cytosol GNMT enzyme activity. There were no significant changes in enzyme activity when liver cytosols were incubated with 1 μ L of 1 mM, 5 mM or 10 mM BaP (in DMSO, final concentrations were 10 μ M, 50 μ M and 100 μ M, respectively), DMSO solvent, or water before the GNMT assay. Adult liver cytosolic protein (250 μ g) was used and reactions were performed with 1 mM SAM and 10 mM glycine (pH 9.0) at 25°C for 30 min. Each reaction was performed five times.

Table 1

Kinetic parameters of glycine N-methyltransferase of liver cytosol from different species.

Species	K _m (SAM ^a) mM	K _m (glycine) mM	Assay Temperature	Reference
<i>Homo sapiens</i>	0.28	12.2	25°C	(Pakhomova et al. 2004)
<i>Homo sapiens</i>	0.27	6.3	30°C	(Ogawa et al. 1993)
<i>Mus musculus</i>	0.18	3.6	25°C	Pakhomova <i>et al.</i> 2004
<i>Rattus norvegicus</i>	0.11	2.0	25°C	Pakhomova <i>et al.</i> 2004
<i>Rattus norvegicus</i>	0.05	0.29	30°C	Ogawa <i>et al.</i> 1993
<i>Oryctolagus cuniculus</i>	0.32	2.9	30°C	Ogawa <i>et al.</i> 1993
<i>Oryctolagus cuniculus</i>	0.2	1	Unspecified	(Kloor et al. 2004)
<i>Oryctolagus cuniculus</i>	0.1	2.2	37°C	Heady and Kerr 1973
<i>Sus scrofa</i>	0.25	11.1	30°C	Ogawa <i>et al.</i> 1993
<i>Fundulus heteroclitus</i>	0.18	3.24	25°C	This study.

^aSAM: S-adenosylmethionine

Table 2

Putative xenobiotic response elements and their locations in the GNMT (zebrafish) promoter regions

Xenobiotic response elements	Locations
AhR ^a	−1574 to −1558
AhR	−1535 to −1524
ARNT ^b -AhR	−1185 to −1174
AhR	−765 to −760
AhR	−738 to −727

Locations numbered back from transcription start site (+1). GenBank Accession No. NW_001880090.1.

^a AhR: Aryl hydrocarbon receptor^b ARNT: Ah receptor nuclear translocator