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BENZO(A)PYRENE INDUCED GLYCINE N-METHYLTRANSFERASE MESSENGER RNA EXPRESSION IN *FUNDULUS HETEROCLITUS* EMBRYOS

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Abstract

Glycine N-methyltransferase (GNMT) is a mediator in the methionine and folate cycles, and is responsible for the transfer of a methyl group from S-adenosylmethionine (SAM) to glycine forming S-adenosylhomocysteine (SAH) and sarcosine. All the known DNA methyltransferases use SAM as a methyl donor thus, GNMT is critically involved in regulation of DNA methylation. Altered GNMT activities have been associated with liver pathologies including hepatocellular carcinoma. The homotetramer form of GNMT is enzymatically active, but the homodimeric form has been suggested as the 4S PAH-binding protein which may mediate CYP1A expression. To further understand the role of GNMT in benzo(a)pyrene (BaP)-related toxicity, full length *Fundulus heteroclitus* GNMT cDNA was cloned from adult liver. The open reading frame (ORF) of GNMT is 888 base pairs long and encodes a deduced protein of 295 amino acids which has 74% identity with human GNMT. Expression of GNMT mRNA was determined by quantitative RT-PCR. In unfertilized, 2 day postfertilization (dpf), and 3 dpf embryos GNMT was constitutively higher than in 4, 7, 10 or 14 dpf embryos. Embryos were also exposed to waterborne BaP at 10 and 100 $\mu\text{g} \cdot \text{L}^{-1}$, and by 10 dpf the higher BaP dose caused increased expression of GNMT mRNA. These results suggest that PAH exposure may alter expression of an important physiological methylation mediator. Future work will be necessary to determine enzyme level effects of BaP exposure as well.

Keywords

Benzo(a)pyrene; GNMT; *Fundulus heteroclitus*

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous marine and aquatic contaminants. BaP exposure specifically can cause both fish embryo toxicity and liver carcinogenesis. While many of BaP's effects appear to be aryl hydrocarbon (AhR)- and CYP1-mediated toxicities (Billiard et al., 2007), we hypothesized that glycine N-methyltransferase (GNMT; EC2.1.1.20) could also be playing a role in PAH toxicity. GNMT is responsible for the transfer of a methyl group from S-adenosylmethionine (SAM) to glycine forming S-adenosylhomocysteine (SAH)

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and sarcosine, preserving the ratio of SAM/SAH, which is a sensitive indicator of methylation status. Altered expression of GNMT or SAM homeostasis is related to liver disease including hepatocellular carcinoma (HCC) and global or chromosome specific DNA methylation changes (Martinez-Chantar et al., 2008; Rowling et al., 2002). The homotetramer form of GNMT is enzymatically active and has unique glycine and folate binding sites. The homodimeric form, however, has been suggested as the 4S PAH-binding protein which may translocate to the nucleus and mediate CYP1A induction by various PAHs independent of the AhR (Bhat & Bresnick, 1997). More recently GNMT's ability to bind BaP was suggested as a protective mechanism in that binding would sequester BaP, and in turn, decrease adduct formation, CYP1A1 and GNMT activity and cytotoxicity (Chen et al., 2004). Previous studies in our laboratory have found that exposures to high concentrations of PAHs are capable of causing embryo toxicity and producing HCC in adult *Fundulus heteroclitus*. In this study, BaP was used to study effects on GNMT mRNA expression in *Fundulus* during embryo development to determine whether altered GNMT expression could be a potential mechanism involved in BaP-associated developmental toxicities.

Fundulus GNMT cDNA was cloned from adult liver mRNA. A partial *Fundulus* GNMT (764 bp) EST was found in the *Fundulus* EST database (FunnyBase, http://genomics.rsmas.miami.edu/funnybase/super_craw4/). Primers were designed based on this partial sequence. Both 5' and 3' RACE were used to amplify the respective ends of the cDNA, and then full length PCR products were generated using primers: forward: 5'-ACTAGCGGCAGCATGTC-3', reverse: 5'-CCCGGATCTCAGATAAATCAG-3'. The full length GNMT cDNA contained a 5' UTR of 63-base pairs (bp) long, and an open reading frame (ORF, Genbank Accession #FJ607947) of 888 bp. Because of sequence differences in the 3' UTR between clones, genomic DNA was used as template to amplify the 3' UTR of GNMT by using forward: 5'-CATGGAGCACACCGTCTAC-3', reverse: 5'-CCCGGATCTCAGATAAATCAG-3' primers. Three copies of the 3' UTR with length of 794, 797 or 804 bp were cloned (data provided as a supplementary figure). *Fundulus* GNMT 3' UTR was much longer compared to human (198 bp, #NM_018960) and zebrafish UTRs (351 bp, #NM_212816.1) and, to date, only *Fundulus* appears to have multiple copies (at least three) of GNMT 3'UTR. The significance of the variation in this region and between species is unknown.

Fundulus GNMT cDNA encodes a deduced protein of 295 amino acids with 74% and 79% identities with human (#Q14749) and zebrafish GNMTs (#AAH62527), respectively. Six single nucleotide polymorphisms (SNP) were discovered in the ORF region from two fish livers (represented by 13 and 9 colonies sequenced, respectively). SNPs in ORF positions 78 (C/T), 273 (A/G), 300 (A/C) did not change the amino acid translation, but SNPs in position 455 (A/T), 587 (A/G) and 841 (A/G) caused amino acid changes (Figure 1). However, as indicated in Figure 1, these amino acid changes were neither located in glycine nor SAM binding sites.

Fertilized eggs from *Fundulus* (caught in an uncontaminated site in the Newport River near Beaufort Inlet, NC, USA) were collected and randomly divided by 4.5 hpf into three waterborne treatment groups, namely control (dimethylsulfoxide, DMSO, 1 μL μL^{-1}), 10 and 100 μg μL^{-1} BaP. Ten embryos per pool ($n = 3 - 6$ pools) per time point were collected at 2, 3, 4, 7, 10, and 14 dpf. After 10 days of exposure, some embryos were returned to clean water prior to collection on day 14. RNA was isolated using Trizol reagent. Abundance of GNMT and 18S rRNA (internal control, #AF021880) transcripts was determined by quantitative real time RT-PCR with SYBR®Green on an ABI7500 using the $2^{-\Delta\Delta C_T}$ method (ABI PRISM 7700 Sequence Detection System User's Manual). Statistical differences between treatments or times were determined on the linearized $2^{-\Delta C_T}$ values. Real time primers for GNMT and 18S rRNA were: GNMT forward: 5'-CCTGCCGGACTTCAAAGGA-3', reverse: 5'-

TACGGTGGTTCGATGATGAGAAT-3'; 18S forward: 5'-TGGTTAATTCCGATAACGAACGA-3', reverse: 5'-CGCCACTTGTCCCTCTAAGAA-3'. Amplification efficiencies of the two primer pairs were not statistically different. In addition to melt curve analyses, the GNMT real time PCR product was confirmed by sequencing.

Unfertilized eggs had the highest expression of GNMT mRNA, suggesting maternal deposition into the egg and a possible important role of GNMT in early development. Constitutive GNMT mRNA expression decreased on 2 and 3 dpf but was still significantly higher than when expression leveled off between 4 and 14 dpf (Figure 2). BaP significantly induced GNMT mRNA expression over controls at 3, 10, and 14 dpf, but not at 2, 4 and 7 dpf (Figure 3). The GNMT induction was sustained even when 10 day BaP-exposed embryos were returned to clean water for another 4 days.

These results confirm that GNMT mRNA is present and inducible by BaP exposure in developing *Fundulus*. Therefore, GNMT may not only be important in fish developmental physiology, but also may represent a novel mechanism involved in PAH toxicity. Further work will be necessary to determine BaP effects on GNMT protein expression and enzyme activity and whether these changes, in turn, can impact DNA methylation status.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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      M S V D S V F R T R S L G V A A E G L P
1  atgtcgggtggacagcgtgttcggaacccgctccctcgggtgtggccgcccaggggctgccc 60
      D Q Y A D G K A A K V W E L Y I G D L Q
61  gaccagtacgccgacggcaaggcggccaaagtgtgggagctgtacatcggagacctccag 120
      S R T Q E Y K S W L V A L L R R H G V R
121  agccggaccaggagtacaagagctggctgggtggccctgctcaggcggcacggcgtgccc 180
      R V L D V A C G T G V D S I M L V E E G
181  aggggtgctggacgtggcctgcggtaccggagttgactccatcatgctgggtggaggagggc 240
      F S L V S V D A S D K M L K Y A L K T R
241  ttcagcctgggtcagcgttgacgccagcgataaaatgctgaagtacgcgtgaagaccgga 300
      W N R R K E A A F D Q W V I E E A N W L
301  tggaaccgcaggaaggaggccgcttcgaccagtgggtgatcgaagaggccaactggctg 360
      T L P E D V E K P A E G F D A V I C L G
361  actctccctgaagacgtggagaaaccggccgaaggcttcgacgccgtcatctgcctgggg 420
      N S F A H L P D F K G D Q S E Q K L A L
421  aactccttcgccacctgccggacttcaaaggagaccagagcgcagcagaagctggctctg 480
      N N I A S M V R P G G I L I I D H R N Y
481  aacaacattgccagcatggtgcggcccgccggttcattcatcatcaccaccgtaactac 540
      D Y I L E T G R A P Q G K N I Y Y K S D
541  gactacatcctggagacgggcccgcgcgcgcagggcaagaacatttactacaagagcgac 600
      L T Q D I S T S V L W V N S K P H M I T
601  ctgaccaggacatcagcacctcggttctgtgggtcaacagcaagccccacatgatcact 660
      L D Y T I Q V P Q A A L Q K L P E V S K
661  ctggactacaccatccaggtgccccaggcggccctgcagaagcttcctgaagtcagtaag 720
      F R L S Y Y P H R L E K F S E L L A D A
721  ttccgcctgtcctactaccctcaccgcctggagaagttcagcgcagctgctggcggacgcc 780
      F S G A M E H T V Y G D F K A Y R P A Q
781  ttcagcggcgccatggagcacaccgtctacggagacttcaaggcctacaggccggcccag 840
      T Q A P C Y F I H V C R R T A *
841  acccaggcgccgtgctacttcatccacgtctgcaggaggaccgcctga 888

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Figure 1.

The open reading frame and deduced amino acid sequences of GNMT from *Fundulus heteroclitus*. Motifs highlighted in grey are the conserved domains of the S-adenosylmethionine binding site (cd02440, NCBI). Gly140, Asn141 and Arg178 (in rectangles) form a hydrogen-bonding network for glycine binding and orientation (Velichkova & Himo, 2005). Nucleotides in grey and amino acid residues underlined were changed by SNPs.

Figure 2. Constitutive GNMT mRNA expression during *Fundulus* embryo development measured by real time PCR. Bars with different letters are significantly different ($p < 0.05$, $n = 3$ pools; ANOVA and Neumann Keuls multiple comparison tests, GraphPad Prism 5.0). Error bars represent standard error (S.E.) of the mean.

Figure 3.

BaP waterborne exposure induced GNMT mRNA expression in *Fundulus* embryos. Each bar represents $n = 3-6$ pools of 10 embryos. Each pool of samples was measured by real time PCR in duplicate. Bars $\pm$ S.E. indicate fold change of GNMT expression normalized to 18S rRNA expression and relative to individual time-point controls (* $p < 0.05$; ** $p < 0.01$, ANOVA and Neumann Keuls multiple comparison tests). 14 dpf^a = Embryos were exposed to BaP for 10 days and returned to clean water for another 4 days.