Journal of Proteomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Embryonic atrazine exposure elicits proteomic, behavioral, and brain abnormalities with developmental time specific gene expression signatures

Katharine A. Horzmann[a,1], Leeah S. Reidenbach[a], Devang H. Thanki[a], Anna E. Winchester[a], Brad A. Qualizza[a], Geoffrey A. Ryan[a], Kaitlyn E. Egan[a], Victoria E. Hedrick[b], Tiago J.P. Sobreira[b], Samuel M. Peterson[a,2], Gregory J. Weber[a], Sara E. Wirbisky-Hershberger[a], Maria S. Sepúlveda[c], Jennifer L. Freeman[a,d,*]

[a] School of Health Sciences, Purdue University, West Lafayette, IN 47907, United States
[b] Bindley Bioscience Center, Discovery Park, Purdue University, West Lafayette, IN 47907, United States
[c] Forestry and Natural Resources, Purdue University, West Lafayette, IN 47907, United States
[d] Purdue Center for Cancer Research, Purdue University, West Lafayette, IN 47907, United States

ARTICLE INFO

Keywords:
Atrazine
Developmental toxicity
Gene expression
Behavior
Proteomics
Zebrafish

ABSTRACT

Atrazine (ATZ), the second most commonly used herbicide in the United States, is an endocrine disrupting chemical linked to cancer and a common drinking water contaminant. This study further investigates ATZ-related developmental toxicity by testing the following hypotheses in zebrafish: the effects of embryonic ATZ exposure are dependent on timing of exposure; embryonic ATZ exposure alters brain development and function; and embryonic ATZ exposure changes protein abundance in carcinogenesis-related pathways. After exposing embryos to 0, 0.3, 3, or 30 parts per billion (ppb) ATZ, we monitored the expression of cytochrome P450 family 17 subfamily A member 1 (*cyp17a1),* glyoxalase I (*glo1*), ring finger protein 14 (*rnf14*), salt inducible kinase 2 (*sik2*), tetratricopeptide domain 3 (*ttc3*), and tumor protein D52 like 1 (*tpd52l1*) at multiple embryonic time points to determine normal expression and if ATZ exposure altered expression. Only *cyp17a1* had normal dynamic expression, but *ttc3* and *tpd52l1* had ATZ-related expression changes before 72 h. Larvae exposed to 0.3 ppb ATZ had increased brain length, while larvae exposed to 30 ppb ATZ were hypoactive. Proteomic analysis identified altered protein abundance in pathways related to cellular function, neurodevelopment, and genital-tract cancer. The results indicate embryonic ATZ toxicity involves interactions of multiple pathways.

Significance: This is the first report of proteomic alterations following embryonic exposure to atrazine, an environmentally persistent pesticide and common water contaminant. Although the transcriptomic alterations in larval zebrafish with embryonic atrazine exposure have been reported, neither the time at which gene expression changes occur nor the resulting proteomic changes have been investigated. This study seeks to address these knowledge gaps by evaluating atrazine's effect on gene expression through multiple time points during embryogenesis, and correlating changes in gene expression to pathological alterations in brain length and functional changes in behavior. Finally, pathway analysis of the proteomic alterations identifies connections between the molecular changes and functional outcomes associated with embryonic atrazine exposure.

1. Introduction

Exposure to environmental stressors, including environmental toxicants, during the developmental period can cause immediate and long lasting health effects [1]. Multiple characteristics of developing organisms, including limited biotransformation of xenobiotics, lack of a blood-brain-barrier, immature immune system, and increased metabolic rate may contribute to greater toxicity during development [2]. As a consequence, toxic effects appear at much lower exposure concentrations in developing organisms compared to the concentration of toxicant required to cause adverse effects in adults. In addition, developmental plasticity is thought to be significant and critical, as

* Corresponding author at: School of Health Sciences, 550 Stadium Mall Dr., West Lafayette, IN 47907, USA.
E-mail address: jfreema@purdue.edu (J.L. Freeman).
[1] Current affiliation: Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849 USA.
[2] Current affiliation: Division of Neurosciences, Oregon National Primate Research Center, Oregon Health & Sciences University, Beaverton, OR 97006 USA.

https://doi.org/10.1016/j.jprot.2018.07.006
Received 29 March 2018; Received in revised form 29 June 2018; Accepted 2 July 2018

perturbations in physiologic pathways during development can result in non- or maladaptive phenotypes of disease [3]. Certain time points in the embryonic period represent critical windows for gene-environment interactions and heightened susceptibility to extrinsic and intrinsic stressors that result in phenotypic alterations [4].

Endocrine disrupting chemicals (EDCs) represent a broad class of chemicals that interfere with the action of hormones. Exposure to EDCs can disrupt normal physiology and homeostasis throughout development and the life course of an organism [5, 6], though organisms appear to have the greatest sensitivity to EDCs during the developmental period [7]. Hormones are critical for the normal growth and development of many organs and tissues, from reproductive organs to the brain. Any disruptions of the hormonal milieu has the potential to cause irrevocable changes in tissue and organ structure or function [8]. In addition to reproductive dysfunction [9], developmental EDC exposure is associated with cancer [10], alterations in innate immune function [11], obesity [12], and altered cognition, including learning and memory [13]. Furthermore, low-dose exposure to EDCs can have significant health implications, as EDCs often have nonmonotonic, U-shaped, inverted U-shaped, or other non-traditional dose response curves [8, 14]. EDCs include chemicals found in plastics and resins, plasticizers, pharmaceuticals, and pesticides [6, 15].

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine; ATZ), is a triazine herbicide used to control broadleaf and select grassy weeds. As of 2012, it was estimated that between 64 and 74 million pounds was used annually in the United States, making ATZ the second most commonly used agricultural pesticide [16]. Post-application rainfall causes ATZ to leach from fields into ground and surface water where it can persist in the environment [17–20]. The heavy use combined with the estimated 146 day half-life of ATZ in groundwater results in ATZ being the most common pesticide detected in agricultural stream water and both agricultural and urban groundwater sources [21]. Due to the presence of ATZ in public water sources, the US Environmental Protection Agency (EPA) regulates the concentration of ATZ in drinking water with a Maximum Contaminant Level of 3 parts per billion (ppb; μg/L) [22]; however, in 2003, the European Union effectively banned ATZ due to concerns over groundwater contamination and environmental persistence [23].

ATZ is a significant environmental toxicant because it is linked to endocrine disruption, cancer, reproductive disorders, birth defects, and altered nervous system function [24]. Epidemiological studies link ATZ exposure to decreased semen quality in Midwestern men [25], increased risk of breast cancer for women living in areas with medium or high exposure [26]; menstrual cycle irregularities [27], and increased prevalence of small-for-gestational-age infants [28]. ATZ exposure in amphibians is associated with abnormal metamorphosis and feminization [29–32], and in rodents, ATZ disrupts the hypothalamic-pituitary-gonadal axis [33–37]. The central nervous system is also a target of ATZ, with ATZ exposure altering dopaminergic and serotonergic neurotransmission as well as neurobehavior [38–43]. Although evidence suggests ATZ is an EDC, the mechanism of action is still under investigation. ATZ does not appear to have intrinsic estrogenic activity and does not bind to the estrogen receptor [44]. Instead, ATZ seems to alter intracellular signaling through the inhibition of type 4 cyclic nucleotide phosphodiesterases (PDE4), resulting in an increase in cyclic adenosine monophosphate (cAMP) and decreased expression of steroidogenic proteins [45–48]. Additionally, ATZ appears to modify the epigenome. ATZ alters microRNA levels [49] and inhibits the activity and expression of DNA methyltransferases, resulting in decreased global DNA methylation [50]. These epigenetic modifications further provide a mechanism of altered gene expression.

The zebrafish (*Danio rerio*) biomedical model has many advantages in toxicological research, including small size, large clutches, easy husbandry, well-characterized and rapid ex vivo development, short generational interval, a sequenced genome, and conserved metabolic pathways [51–55]. A previous study from our laboratory characterized the effects of embryonic, environmentally relevant, low-dose ATZ exposure on the growth and development and the transcriptome of larval zebrafish and found alterations in head length and disruptions of gene pathways associated with neuroendocrine system development and function, reproductive system development and function, and carcinogenesis [56]. This study further investigates the effects of embryonic ATZ exposure by evaluating how gene expression normally changes over a developmental time course and how ATZ exposure alters gene expression at specific developmental time points, how ATZ effects brain development and behavior, and how ATZ changes the proteome in larval zebrafish. We expect that embryonic ATZ exposure dynamically alters the expression of select genes during critical windows for toxicity. To test this hypothesis, six genes were chosen from the previous study that had altered gene expression at 72 h post fertilization (hpf; the end of embryogenesis) as a result of embryonic ATZ exposure [56]. The genes are cytochrome P450 family 17, subfamily A, member 1 (*cyp17a1),* glyoxalase I (*glo1*), ring finger protein 14 (*rnf14*), salt inducible kinase 2 (*sik2*), tetratricopeptide domain 3 (*ttc3*), and tumor protein D52 like 1 (*tpd52l1*). Each of the genes had altered expression in at least two of the ATZ treatments (3 ppb and 30 ppb) and are associated with cancer (*cyp17a1*, *glo1, rnf14, sik2, ttc3* and *tpd52l1*), the central nervous system (*cyp17a1*, *glo1*, *sik2*, and *ttc3*), and/or the endocrine system (*cyp17a1, glo1, rnf14, sik2, ttc3,* and *tpd52l1*) [56]. The normal expression of these genes was monitored throughout embryogenesis and the effects of embryonic ATZ exposure evaluated at each developmental time point (24, 36, 48, 60, and 72 hpf). We also hypothesize that embryonic ATZ exposure alters the neurodevelopment of larval zebrafish. We measured brain length and behavioral responses to a visual motor response test to evaluate brain morphology and function. Finally, we performed a proteomic analysis to identify differences in protein levels resulting from embryonic ATZ exposure. We hypothesized that pathways associated with cancer, neurological disease, reproductive system disease, and cell cycle and proliferation, which were previously altered in transcriptomic analysis, would also have altered protein levels. By performing a proteomic analysis we aim to link changes in protein levels to behavioral alterations, changes in brain morphology, and changes in gene expression throughout development.

2. Materials and methods

2.1. Zebrafish husbandry and treatment

Embryos were obtained from a breeding colony of wild-type AB strain laboratory zebrafish (*Danio rerio*). Adult zebrafish are maintained in a *Z*-Mod System (Aquatic Habitats, Apopka, FL) on a 14:10 light-dark cycle. Water is maintained at 28 °C, the pH at 7.0–7.3, and salinity at 470–550 μS conductivity. Fish and aquaria are monitored twice daily and fed a mixture of brine shrimp (*Artemia franciscana*; Artemia International LLC., Fairview, Texas), Golden Pearls 500–800 μm (Artemia International LLC., Fairview, Texas), and Zeigler adult zebrafish food (Zeigler Bros Inc., Gardners, PA). Adult zebrafish were bred in spawning tanks according to established protocols [57, 58] and embryos collected immediately after the breeding interval, approximately at the 4–8 cell stage of embryonic development. The embryos were rinsed, randomly sorted into treatment groups, exposed to 0 (filtered aquaria water), 0.3, 3, or 30 ppb (μg/L) ATZ and incubated at 28.5 °C. The ATZ solutions were prepared from aliquots of a stock solution of technical grade atrazine (98.1% purity) (CAS 1912–24-9; Chem Service, West Chester, PA) as previously described [43, 56]. ATZ treatment concentrations were confirmed with an US EPA approved immunoassay kit (Abraxis Atrazine ELISA Kit, Warminster, PA) as previously described [59, 60]. Unless collected beforehand, larvae were rinsed at 72 h post fertilization (hpf) with aquaria water to end ATZ exposure and then maintained in clean aquaria water until collected. All protocols were approved by the Purdue University Animal Care and Use Committee and all fish treated humanely with regard to prevention