Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 27 (2016) 211–218

Journal of Nutritional Biochemistry

Mono-2-ethylhexyl phthalate (MEHP) alters histiotrophic nutrition pathways and epigenetic processes in the developing conceptus

Karilyn E. Sant[a], Dana C. Dolinoy[a], Joseph L. Jilek[a], Brian J. Shay[b], Craig Harris[a,*]

[a]Department of Environmental Health Sciences, University of Michigan, Ann Arbor, Michigan, 48109-2029
[b]Department of Pharmacology, Biomedical Mass Spectrometry Facility, University of Michigan, Ann Arbor, Michigan, 48109-5632

Received 8 May 2015; received in revised form 3 September 2015; accepted 4 September 2015

Abstract

Histiotrophic nutrition pathways (HNPs) are processes by which the organogenesis-stage conceptus obtains nutrients, amino acids, vitamins and cofactors required for protein biosynthesis and metabolic activities. Nutrients are captured from the maternal milieu as whole proteins and cargoes via receptor-mediated endocytosis in the visceral yolk sac (VYS), degraded by lysosomal proteolysis and delivered to the developing embryo (EMB). Several nutrients obtained by HNPs are required substrates for one-carbon (C_1) metabolism and supply methyl groups required for epigenetic processes, including DNA and histone methylation. Increased availability of methyl donors has been associated with reduced risk for neural tube defects (NTDs). Here, we show that mono-2-ethylhexyl phthalate (MEHP) treatment (100 or 250 μM) alters HNPs, C_1 metabolism and epigenetic programming in the organogenesis-stage conceptus. Specifically, 3-h MEHP treatment of mouse EMBs in whole culture resulted in dose-dependent reduction of HNP activity in the conceptus. To observe nutrient consequences of decreased HNP function, C_1 components and substrates and epigenetic outcomes were quantified at 24 h. Treatment with 100-μM MEHP resulted in decreased dietary methyl donor concentrations, while treatment with 100- or 250-μM MEHP resulted in dose-dependent elevated C_1 products and substrates. In MEHP-treated EMBs with NTDs, H3K4 methylation was significantly increased, while no effects were seen in treated VYS. DNA methylation was reduced in MEHP-treated EMB with and without NTDs. This research suggests that environmental toxicants such as MEHP decrease embryonic nutrition in a time-dependent manner and that epigenetic consequences of HNP disruption may be exacerbated in EMB with NTDs.

Keywords: Embryonic development; Neural tube defects; DNA methylation; Histone methylation; Histiotrophic nutrition; One-carbon metabolism

1. Introduction

The processes by which mammalian embryos (EMBs) obtain nutrients and substrates for growth and development during embryogenesis and early organogenesis are known as *histiotrophic nutrition pathways* (HNPs). Because the placenta has not yet become fully functional at this stage, the active maternal–embryonic exchange of micronutrient substrates and metabolic cofactors from maternal circulation is not yet possible. HNPs facilitate the receptor-mediated endocytosis (RME) of bulk and carrier proteins along with their bound nutrient and cofactor cargoes through the visceral yolk sac (VYS) brush border. Subsequent proteolysis of these proteins in secondary vesicles (lysosomes) releases nutrient cargoes required for various cellular processes and supplies nearly 100% of all amino acids required for protein biosynthesis and enzymatic function in the VYS and in the EMB proper [1,2].

HNPs are responsible for providing and transporting many substrates to the EMB, including methyl donors for one-carbon (C_1) metabolism such as methionine, folate, choline and betaine [3–11]. C_1 metabolism is the process by which dietary methyl donors are utilized to synthesize S-adenosylmethionine (SAM), the primary molecular methyl donor for cells. Through C_1 pathway activity, SAM is made available for numerous processes, including posttranslational modifications and epigenetic regulation. Thus, the nutritional state of the conceptus likely influences epigenetic reprogramming and regulation of genetic processes [12].

During the first trimester of pregnancy, cells are rapidly dividing to facilitate overall growth and anatomical form in order to complete embryogenesis. During this time, DNA undergoes substantial epigenetic programming, from near-complete loss of methylation marks after fertilization to reprogramming of methylation marks during embryogenesis and early organogenesis [13,14]. Histone methylation, on the other hand, is variable; methylation increases over developmental time at the histone 3 lysine 4 (H3K4) locus and decreases at the histone 3 lysine 27 (H3K27) locus [15]. Because these processes occur during the phase of HNP-supplied nutrition and provide the necessary methyl donors for epigenetic marks, it is possible that perturbation of HNPs may result in abnormal epigenetic programming. Previous work has demonstrated that inhibition of HNP lysosomal function with leupeptin resulted in decreased DNA methylation in the EMB and VYS during early organogenesis [16].

Common dietary methyl donors and cofactors for C_1 metabolism, including folate, betaine, choline and vitamin B_{12}, are required for

* Corresponding author at: Toxicology Program, Department of Environmental Health Sciences, University of Michigan, 1420 Washington Heights, Ann Arbor, Michigan, 48109-2029. Tel.: +1-734-936-3397; fax: +1-734-763-8095.
E-mail address: charris@umich.edu (C. Harris).

http://dx.doi.org/10.1016/j.jnutbio.2015.09.008
0955-2863/

normal growth and development. Maternal deficiencies in folate and choline during pregnancy have been associated with increased risk for neural tube defects (NTDs) [17–19]. Likewise, folate supplementation of maternal diet, via the fortification of foods or prenatal vitamins, has been associated with nearly a 30% decreased risk for NTDs [20–23]. However, the mechanism by which folate is beneficial is unknown [20,24].

Mono-2-ethylhexyl phthalate (MEHP) is the primary metabolite of the ubiquitous plasticizing agent di-2-ethylhexyl phthalate (DEHP), a compound that has been found almost ubiquitously in consumer, industrial and medical products including PVC products, medical tubing and children's toys. Numerous studies investigating exposures to DEHP have found detectable levels of its metabolites in a great majority of individuals sampled, including pregnant women [25–27]. Previous studies have demonstrated that MEHP exposure during early organogenesis increased the number of EMBs with NTDs [28,29]. Thus, the purpose of this exploratory work is to identify whether this increased number of NTDs due to MEHP exposure is concordant with decreased nutrient bioavailability to the EMB and whether this may have epigenetic consequences. It is hypothesized that MEHP exposure will reduce the activity of HNPs, decreasing the availability of methyl donors and C_1 metabolism, and that these changes will result in decreased DNA and histone methylation.

2. Materials and methods

2.1. Chemicals and reagents

Bicinchoninic acid (BCA), dimethyl sulfoxide (HPLC grade), Tyrode's balanced salt solution (TBSS), penicillin/streptomycin (10,000-units/ml penicillin, 10,000-μg/ml streptomycin sulfate), choline chloride, betaine, folic acid, D,L-homocysteine, D,L-methionine, L-cysteine hydrochloride, N,N,N-Trimethyl-d_9-Glycine HCl (Betaine-d_9) and Homocysteine-d8 were obtained from Sigma/Aldrich (St. Louis, MO, USA). L-Methionine (methyl-d3) and D,L-cysteine (3,3-d2) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA), and S-Adenosyl-L-methionine-d3 (S-methyl-d3) tetra (p-toluenesulfonate) salt was from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Hanks balanced salt solution (HBSS) was purchased from GIBCO/Life Technologies (Grand Island, NY, USA). MEHP was obtained from AccuStandard (New Haven, CT, USA).

2.2. Mouse whole EMB culture

Mouse embryo culture was performed following the conditions outlined in Ref. [30] and described in Ref. [29]. Briefly, female CD-1 mice were time mated and obtained from Charles River (Portage, MI, USA). The morning of a positive vaginal smear was designated as gestational day (GD) 0. Animals were maintained on a 12-h light–12-h dark cycle and were supplied food and water *ad libitum*. Pregnant mice were euthanized with CO_2, supplied at 10–30% on GD 8, and the uterus was removed. Conceptuses were explanted from the uterus, randomized and placed into 10-ml culture bottles. Each culture bottle contained 5 ml of 75% heat-inactivated rat serum/25% TBSS and 21.5-μl penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA; 10,000-U/ml penicillin; 10,000-μg/streptomycin sulfate). Bottles were placed on a continuous-gassing carousel and initially supplied with 5% O_2/5% CO_2/90% N_2. The gas mixture was changed after 6 h to 20% O_2/5% CO_2/75% N_2 to optimize conceptal growth. All experiments were performed in strict concordance with protocols approved by the University Committee on Use and Care of Animals.

2.3. Exposures and sample collection

MEHP was suspended in DMSO to increase solubility and stored at a concentration of 1 mg/ml. This MEHP mixture was diluted in DMSO and added to each treatment culture bottle to produce a final concentration of 100 or 250 μg/ml. These concentrations were optimized from previous experiments [29], based on viability and morphology, as well as reproducibility from other phthalate WEC experiments [28,31]. Control bottles received an equivalent volume of DMSO only, and the total volume added was no greater than 1-μl/ml culture medium. Conceptuses remained in culture for 24 h.

Following the culture period, conceptuses for DNA and histone analysis were thoroughly rinsed in HBSS and briefly inspected, at which time anatomical malformations and delayed closure of the embryonic neural tube were noted. EMBs with closed neural tubes were denoted as NTD (−) while those with open neural tubes were denoted as NTD (+) (Fig. 1). At the time of collection, no control samples were found to have abnormal neural tube closure. Conceptuses providing tissues for DNA isolation were dissected into EMB and VYS, snap frozen and stored at −76 °C until use. Conceptuses for histone isolation were also dissected into EMB and VYS, but histone proteins were immediately extracted using the EpiQuik™ Total Histone Extraction Kit (Epigentek). Following extraction, proteins were stored at −20 °C overnight until completion of histone methylation experiments the following day.

Conceptuses used for HNP analysis were randomized and explanted into culture on GD 8. After 24 h in culture (GD 9), fluorescence-tagged FITC-albumin was added to the culture media at a nontoxic total concentration of 100 μg/ml [32,33]. At this time, 100- or 250-μg/ml MEHP was added. Treated conceptuses were returned to the culture apparatus and grown in complete darkness for 3 h. At the conclusion, conceptuses were removed from culture and thoroughly rinsed in HBSS. Ethanol (6 mg/ml) was added to concurrent cultures, and HNP assays were performed as a positive control because it is a known inhibitor of endocytosis in the HNP [34,35]. Following culture, conceptuses were pooled in duplicate and transferred into a 150-μl drop containing 50-mM sodium phosphate buffer (pH = 6.0) for dissection. The VYS was first removed and collected into 0.1% Triton X-100, followed by the EMB. The remaining extra-embryonic fluids (EEFs) were then released into the sodium phosphate buffer and also collected.

Whole conceptuses for analysis for C_1 component quantification or quantification of methyl donors were treated with 100 or 250-μg/ml MEHP on GD 8 and grown for 24 h in culture. At the conclusion of the culture period, whole conceptuses were carefully rinsed and stored intact in polypropylene tubes at −80°C for later analysis. Samples for analysis of C_1 components and for choline and betaine were quantitated as described below using two separate by LC-MS/MS techniques. Conceptuses for immunoassay quantification of folate and vitamin B_{12} were collected into 500-μl 6% trichloroacetic acid (TCA).

2.4. Histiotrophic nutrition assay

Histiotrophic nutrition assays were conducted using a modified version of the protocol outlined in [32]. Even though its contribution to total fluorescence is usually minimal, fluorescence in the EMB fraction of the conceptus was also measured. After collection of tissues and fluids, samples were sonicated to mix. A 20-μl aliquot of each sample and of culture media were immediately collected and set aside for measurement of total fluorescence, following addition of 230 μl of Triton X-100 and 750 ml of 6% TCA with 1% SDS. Seven hundred fifty microliter of 6% TCA was added to the remainder of each sample, after which they were vortexed and allowed to precipitate at 4 °C overnight.

The next morning, samples were centrifuged at 9500×*g* for 10 min. The supernatant of each sample was transferred to a new tube for quantification of soluble fraction fluorescence. One ml of 500 mM and 150 μl of 1-N sodium hydroxide were added to each tube before setting aside for fluorescence quantification. The insoluble fractions were precipitated in 150 μl of 1-N NaOH at room temperature for 1 h, prior to addition of Tris buffer, sodium hydroxide and 6% TCA in equivalent amounts to the soluble fractions.

Soluble, insoluble and total fluorescence were quantified by fitting sample fluorescence to a standard curve, ranging from 1- to 250-μg/ml FITC-albumin. Samples were plated onto opaque, black 96-well plates in triplicate replicates of 200 μl for each sample. Plates were read by spectrofluorometer, with excitation and emission wavelengths set at 495 and 520 nm, respectively. Normalization of results to sample protein concentrations was performed following BCA assay. *Clearance*, defined as the volume of culture media cleared of FITC-albumin per minute per milligram of conceptal protein, was calculated for each fraction and tissue, and the cumulative total clearance was also determined by adding the clearance values from all measured compartments.

2.5. C_1 metabolism component quantification

Quantification of C_1 components was performed as previously described in Ref. [16]. Liquid chromatography-tandem mass spectrometry was performed using a TSQ Quantum Ultra AM triple quadrupole mass spectrometer (Thermo Scientific, Rockford, IL, USA) coupled to a Waters 2695 Separations Module (Waters, Milford, MA, USA). These were fitted with a Luna C18(2) 5-μM 250×4.6-mm column and 4-mm C18 guard column (Phenomenex, Torrence, CA, USA). All data were recorded using Xcalibur software (Thermo Scientific).

Briefly, isotopically labeled internal standards (ISTD) for SAM, methionine, cysteine and homocysteine were added to each sample in known quantities following the incubation in reducing agent. To precipitate protein in the samples, 0.1% formic acid and 0.05% trifluoroacetic acid in acetonitrile were added to the samples before centrifugation, and transfer of the supernatant to high-performance liquid chromatographic (HPLC) vials and 10 μl was injected in the positive ion electrospray LC-MS/MS analysis. The pellet was resolubilized in 0.25-M sodium hydroxide, and total protein was quantified using BCA assay. The gradient method was programmed to flow 1 ml/min of water 0.1% formic acid changing linearly to 10% acetonitrile 0.1% formic at 10 min, then back to initial conditions and held until 30 min. All ion source and precursor/fragment ion transitions were optimized by direct infusion experiments. Concentrations of each analyte were calculated by ratio of the measured analyte to the known quantity of the isotopic standard in each sample. All data were normalized to the total protein concentration of the conceptus to account for the differences in size and morphology due to MEHP treatment [16,29].

2.6. Quantification of choline and betaine

Quantification of choline and betaine was performed using a modified version of the protocol outlined in [36]. Briefly, 100-ul water was added to tubes containing each conceptus, and the contents were tip sonicated for suspension. The conceptus volume