Reproductive Toxicology 63 (2016) 32–48

Contents lists available at ScienceDirect

Reproductive Toxicology

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

Mono-2-ethylhexyl phthalate disrupts neurulation and modifies the embryonic redox environment and gene expression

Karilyn E. Sant[a], Dana C. Dolinoy[a,b], Joseph L. Jilek[a], Maureen A. Sartor[c,d], Craig Harris[a,b,*]

[a] Department of Environmental Health Sciences, University of Michigan School of Public Health, Ann Arbor, MI 48109, United States
[b] Department of Nutritional Sciences, University of Michigan School of Public Health, Ann Arbor, MI 48109, United States
[c] Department of Computational Medicine & Bioinformatics, University of Michigan Medical School, Ann Arbor, MI 48109, United States
[d] Department of Biostatistics, University of Michigan School of Public Health, Ann Arbor, MI 48109, United States

ARTICLE INFO

Article history:
Received 14 September 2015
Received in revised form 9 February 2016
Accepted 28 March 2016
Available online 7 May 2016

Keywords:
Embryo
Neural tube defects
MEHP
DEHP
Visceral yolk sac
Redox potential
Glutathione
Cysteine
Gene expression

ABSTRACT

Mono-2-ethylhexl phthalate (MEHP) is the primary metabolite of di-2-ethylhexyl phthalate (DEHP), a ubiquitous contaminant in plastics. This study sought to determine how structural defects caused by MEHP in mouse whole embryo culture were related to temporal and spatial patterns of redox state and gene expression. MEHP reduced morphology scores along with increased incidence of neural tube defects. Glutathione (GSH) and cysteine (Cys) concentrations fluctuated spatially and temporally in embryo (EMB) and visceral yolk sac (VYS) across the 24 h culture. Redox potentials (E_h) for GSSG/GSH were increased by MEHP in EMB (12 h) but not in VYS. CySS/CyS E_h in EMB and VYS were significantly increased at 3 h and 24 h, respectively. Gene expression at 6 h showed that MEHP induced selective alterations in EMB and VYS for oxidative phosphorylation and energy metabolism pathways. Overall, MEHP affects neurulation, alters E_h, and spatially alters the expression of metabolic genes in the early organogenesis-stage mouse conceptus.

1. Introduction

Development is a highly regulated process that relies on tightly controlled intra- and intercellular signaling for normal growth. Dysregulation of embryogenesis can be caused by a myriad of genetic, physiological, and environmental factors such as the chemicals found in our air, water, and food. Exposure to chemical agents can alter specific signaling pathways to manipulate cell polarity and migration, membrane transport, and protein structure and function [1–4]. Ultimately, these changes can manifest as spontaneous abortions, birth defects, and growth deficits or predispose individuals to diseases that arise during childhood and into adulthood. One such factor that can lead to this dysregulation is altered redox signaling and control, caused by perturbations of the soluble thiol steady state, along with an increase in reactive oxygen species (ROS) [5]. Many human and animal teratogens, compounds that produce congenital malformations, are reported to act through classical oxidative stress: a change from a balanced and reducing intracellular redox environment to predominantly oxidizing conditions, concurrent with the generation of ROS [6]. An oxidizing environment during development has also been associated with increased risk of postnatal chronic diseases including neurodegeneration, hypertension, cancer, and Type II Diabetes [7–9]. Environmental contaminants, including the phthalates discussed in this report, have also been shown to increase the generation of ROS [10–13]. Views about the roles of increased ROS generation in biological cells and tissues have changed of late because ROS are now known to have critical signaling and regulatory roles in normal cell function [14–16]. These discoveries have helped dispell the previous notion that all ROS are deleterious. Through the selective oxidation and reduction of protein thiols, critical cell processes such as enzyme activity, receptors, transporters, transcription factors, and second messenger signaling are all regulated. While related, the widespread practice of invoking "oxidative stress" through the simple measurement of glutathione (GSH) depletion or increases in markers of lipid peroxidation because they vary naturally and do not represent the significance of the

* Corresponding author at: Department of Environmental Health Sciences, University of Michigan School of Public Health, Ann Arbor, MI 48109, United States.
E-mail address: charris@umich.edu (C. Harris).

http://dx.doi.org/10.1016/j.reprotox.2016.03.042
0890-6238/

broader redox environment [17]. Many of the regulatory consequences of altered redox status have been more accurately assessed by measuring intracellular redox potentials (E_h). which, although based on the GSSG/GSH redox couples, are a more sensitive indicator of environmentally-induced perturbations to the redox steady state. Progressive increases in intracellular E_h are highly correlated with changes in proliferation, differentiation, and tissue patterning during embryogenesis and organogenesis [5,18]. Altered E_h during embryonic development can range from mild to severe, leading to manifestations ranging from distorted signal transduction to apoptosis and necrosis, respectively. Because these consequences are likely harmful, the body has an endogenous and dynamic antioxidant system to prevent and protect against the more damaging consequences of oxidation. The reducing agent glutathione (GSH) and related enzymes glutathione reductase, glutathione peroxidase, and glutamate-cysteine ligase are primarily responsible for the maintenance of a balanced cellular E_h [17]. Production and recycling of these endogenous antioxidants is regulated through the induction of the Nrf2 antioxidant pathway [19]. The action of other redox couples, such as reduced cysteine (Cys) and oxidized cysteine (CySS) or thioredoxin, help maintain steady redox states and govern redox signaling in the embryo. When the actions of all of these antioxidants and protective enzymes are insufficient to maintain a balanced environment during development, teratogenesis can occur [5].

Mono-2-ethylhexyl phthalate (MEHP) is the primary metabolite of the common plasticizing agent, di-2-ethylhexyl phthalate (DEHP). DEHP has been widely incorporated into many products worldwide, but is of special concern because of its inclusion in vinyl piping and medical tubing. Evidence of phthalate transfer to the EMB and fetus has been evident through detection of MEHP in meconium, amniotic fluid, cord blood, rodent fetal tissues, and placental perfusate [20–28]. It has been previously demonstrated that MEHP can induce general oxidative stress and inflammation in various reproductive tissues and developmental tissues *via* the increased generation of ROS, though these studies were conducted in other model organisms and at different stages of development [12,13,29,30]. It has been demonstrated that several chemical compounds have the ability to modify the embryonic redox environment, and that these changes can result in teratogenic outcomes [5,31–35]. Compounds such as ethanol, diamide, methylmercury, phenytoin, and L-buthionine-S,R-sulfoximine have been associated with oxidation of the various tissue and fluid compartments of the conceptus, including VYS, the yolk sac fluid (YSF), amniotic fluid (AF), and the EMB [34,36–38]. These compounds are able to decrease GSH concentrations and selectively increase Eh in conceptal tissues and fluids. The body of evidence for other toxicants altering redox signaling in the organogenesis-stage conceptus is growing, and many of these exposures are associated with adverse health outcomes and structural defects.

Thus, it was important to determine the ontogeny of E_h across the susceptible window of early organogenesis in order to identify potential windows of susceptibility for phthalate exposure and relate disturbances to sensitive developmental events. The goals of this study were to characterize MEHP-induced morphology and growth changes in mouse whole embryo culture (mWEC), determine whether MEHP alters conceptal E_h inEMB and VYS tissues, and to identify related MEHP-induced changes in gene expression.

2. Materials & methods

2.1. Chemicals and reagents

Mono-2-ethylhexyl phthalate was obtained from AccuStandard (New Haven, CT). Dioctyl phthalate (DEHP), dimethyl sulfoxide (DMSO), glutathione, glutathione disulfide, cysteine, cystine, γ-glutamyl-glutamate, iodoacetic acid, iodoacetamide, bicinchoninic acid, RNAlater®, Tyrode's balanced salt solution, and penicillin/streptomycin (10,000 units/ml penicillin, 10,000 μg/ml streptomycin sulfate) were obtained from Sigma/Aldrich (St. Louis, MO). Hanks balanced salt solution (HBSS) was purchased from GIBCO/Life Technologies (Grand Island, NY). Dansyl chloride was purchased from Fluka Chemie/Sigma-Aldrich (St. Louis, MO). Glacial acetic acid was obtained from Fisher Scientific (Waltham, MA).

2.2. Mouse whole embryo culture

Mouse embryo culture was performed according to the procedures outlined in Ref. [39]. Briefly, female CD-1 mice were time-mated and obtained from Charles River (Portage, MI). The morning of discovery of a vaginal plug 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*. On GD 8, female mice were euthanized with CO_2 (10–30%) and the uterus was removed. Culture-ready conceptuses (ranging from 6 to 8 somites) were explanted from the uterus, removed from the decidual mass, freed from the Reichert's membrane, randomized, and placed into 10 ml culture bottles containing 5 ml of 75% heat-inactivated rat serum/25% Tyrode's balanced salt solution (TBSS) and 21.5 μl penicillin/streptomycin. Immediately centrifuged rat serum was collected and prepared from female Sprague Dawley rats according to approved protocols [39]. The number of conceptuses per bottle never exceeded the standard rule of 1 conceptus per ml of culture medium. Bottles were placed on a continuous-gassing carousel in an incubator held at 37 °C and supplied with 5% O_2/5% CO_2/90% N_2. After 6 h in culture, the gas input was changed to 20% O_2/5% CO_2/75% N_2 to optimize growth in culture. All animal procedures were approved by the University of Michigan University Committee on Use and Care of Animals.

2.3. Exposure and sample collection

DEHP and MEHP were suspended in DMSO to increase solubility in culture. DEHP and MEHP were added to the culture medium to bring the final concentration in culture to 100, 250, 500, or 1000 μg/ml (0.4–3.6 mM MEHP and 0.3-2.6 mM DEHP). These concentrations were selected based upon concentrations utilized in other phthalate whole embryo culture studies [40,41]. WEC experiments, like cell culture experiments, frequently use concentrations greater than those observed *in vivo* due to the lack of direct perfusion to the tissues, and thus these concentrations are 1–3 orders of magnitude greater than those observed in human cord blood [27,28]. DMSO was added to control bottles at a concentration of 0.05% (v/v) in culture, equal to the volume of added DEHP and MEHP solutions. For morphology experiments, conceptuses were grown in culture for a total of 24 h before removal. For redox analysis, conceptuses were sampled at 0 h (before explant into culture), and at 1, 3, 6, 12, and 24 h of culture. Samples designated for RNA isolation were collected following 6 h in culture. At the time of removal from culture, all conceptuses were washed 2X in Hank's balanced salt solution (HBSS) and ectoplacental cones were removed. The EMB and VYS were manually separated using watchmaker's forceps. At this time, samples were either designated for morphology assessment or for redox analysis. Individual (unpooled) samples for redox analysis were placed into thiol preservation buffer (containing 5% perchloric acid, 0.2 M boric acid, and 10 μM γ-glutamylglutamate), snap frozen in liquid N_2, and stored at −76 °C, as specified in [18]. Tissues collected for RNA isolation were pooled (n = 5 per sample) and placed into RNA later and stored at −76 °C.