Reproductive Toxicology 78 (2018) 9–19

Contents lists available at ScienceDirect

Reproductive Toxicology

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

Intergenerational response to the endocrine disruptor vinclozolin is influenced by maternal genotype and crossing scheme

Edward W. Pietryk[a], Kiristin Clement[b], Marwa Elnagheeb[a], Ryan Kuster[b], Kayla Kilpatrick[c], Michael I. Love[a,c], Folami Y. Ideraabdullah[a,b,d,*]

[a] Department of Genetics, School of Medicine, University of North Carolina at Chapel Hill, 120 Mason Farm Rd, CB#7264, Chapel Hill, NC 27599, United States
[b] Nutrition Research Institute, University of North Carolina at Chapel Hill, 500 Laureate Way, Kannapolis, NC 28081, United States
[c] Department of Biostatistics, Gillings School of Public Health, University of North Carolina at Chapel Hill, 135 Dauer Drive, CB #7420, Chapel Hill, NC 27599, United States
[d] Department of Nutrition, Gillings School of Public Health, University of North Carolina at Chapel Hill, 135 Dauer Drive, CB #7461, Chapel Hill, NC 27599, United States

ARTICLE INFO

Article history:
Received 17 October 2017
Received in revised form 5 March 2018
Accepted 6 March 2018
Available online 10 March 2018

Keywords:
Epigenetic inheritance
Endocrine disruptor
Vinclozolin
Gene-environment
Maternal effect
Genomic imprinting
DNA methylation

ABSTRACT

In utero exposure to vinclozolin (VIN), an antiandrogenic fungicide, is linked to multigenerational phenotypic and epigenetic effects. Mechanisms remain unclear. We assessed the role of antiandrogenic activity and DNA sequence context by comparing effects of VIN vs. M2 (metabolite with greater antiandrogenic activity) and wild-type C57BL/6 (B6) mice vs. mice carrying mutations at the previously reported VIN-responsive *H19/Igf2* locus. First generation offspring from VIN-treated 8nrCG mutant dams exhibited increased body weight and decreased sperm *ICR* methylation. Second generation pups sired by affected males exhibited decreased neonatal body weight but only when dam was unexposed. Offspring from M2 treatments, B6 dams, 8nrCG sires or additional mutant lines were not similarly affected. Therefore, pup response to VIN over two generations detected here was an 8nrCG-specific maternal effect, independent of antiandrogenic activity. These findings demonstrate that maternal effects and crossing scheme play a major role in multigenerational response to *in utero* exposures.

1. Introduction

Endocrine-disrupting compounds (EDCs) are environmental compounds that interfere with the homeostasis of the endocrine system. EDC exposure is of growing concern because it is linked to increased prevalence of human diseases such as cancer, diabetes, obesity, asthma, neurodegenerative disorders, and reproductive disorders [1]. Furthermore, experimental models show that exposure to EDCs *in utero* can negatively influence offspring health outcomes over multiple generations [2]. Vinclozolin (VIN) is a dicarboximide fungicide that is still used commercially but in limited application in the United States (US) due to adverse effects on male reproduction detected in experimental models [3–5]. In rodents, VIN exposure *in utero* disrupts male reproduction causing reduced anogenital distance (AGD) and sperm count, cleft phallus, hypospadias, ectopic testes, vaginal pouches, and epididymal granulomas [6–8].

The mechanism by which VIN perturbs male reproduction is not fully understood. However, VIN and two of its metabolites (M1 & M2) have been shown to compete with dihydrotestosterone (DHT) for binding the androgen receptor (AR) and therefore may act in part by disrupting genomic activity of AR [9,10]. Compared to VIN and M1, M2 seemingly has greater potential to interfere with AR activity. M2 was previously reported to have a greater ability to compete with androgen for binding to AR (DHT (K_i = 100 nM) > hydroxyflutamide (K_i = 175 nM) > testosterone (K_i = 220 nM) >M2 (K_i = 9.65 μM) > M1 (K_i = 92 μM) > VIN (K_i > 700 μM)) [11,12]. M2 was also shown to

Abbreviations: VIN, Vinclozolin, or 3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione; EDC, Endocrine disrupting compound; M1, 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid; M2, 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide; AR, Androgen Receptor; ER, Estrogen Receptor; PR, Progesterone Receptor; GD, Gestational Day; PND, Postnatal day; AGD, Anogenital distance; DHT, Dihydrotestosterone; SRY, Sex determining region of the Y chromosome gene; ICR, Imprinting Control Region; BWS, Beckwith-Wiedemann syndrome; DMR, Differentially methylated region; G_0, Generation zero, parental generation; G_1, First generation; G_2, Second generation.

* Corresponding author at: Department of Genetics, School of Medicine, University of North Carolina at Chapel Hill, 120 Mason Farm Rd, CB#7264, Chapel Hill, NC 27599.
E-mail address: folami@email.unc.edu (F.Y. Ideraabdullah).

https://doi.org/10.1016/j.reprotox.2018.03.005
0890-6238/

be a more potent antagonist of AR transcriptional activity, acting at a dose that was more similar to hydroxyflutamide (a similarly structured known antiandrogenic compound) [9]. VIN and its metabolites have also been reported to interact with other steroid receptors. Here also, M2 was shown to be a more potent antagonist of transcriptional activity of progesterone receptor (PR) and mineralocorticoid receptor (MR) and a more potent agonist of AR and estrogen receptors (ER) α and β [13–15].

VIN exposure *in utero* was the first reported model of transgenerational epigenetic inheritance. Male rats exposed to VIN *in utero* exhibited adverse reproductive outcomes and altered DNA methylation in sperm over 3 generations [8,16]. A separate study also reported transgenerational DNA methylation changes at imprinted loci including the *H19/Igf2* imprinting control region (ICR) in mature sperm [17]. Genetic differences have been shown to influence heritable responses to VIN in reports showing that transgenerational germline epimutations could be detected in outbred CD-1 mice but not inbred 129 mice [18]. Exact sequences responsible were not investigated. The mechanism of persistence of VIN-induced phenotypic and epigenetic changes are unclear. However, recent studies show that VIN-induced methylation changes do not persist through stages of reprogramming during male germ cell development [19] suggesting an alternate mechanism of transmission.

Here, we used the VIN mouse exposure model to investigate factors that may contribute to differences in sensitivity to endocrine disruptors. We used a two-stage study design to examine the role of M2 metabolite availability (compared to VIN) and DNA sequence context (wildtype vs. mutations at the *H19/Igf2* ICR) in determining the extent and heritability of epigenetic and phenotypic outcomes in response to VIN. The mutant lines used, 8nrCG, Δ2,3 and ΔIVS, carry targeted mutations at the *H19/Igf2* ICR that mimic genetic mutations found at the epigenetically perturbed human locus in patients with the overgrowth disorder Beckwith-Wiedemann syndrome (BWS) [20–26]. Aberrant *H19/Igf2* expression is also associated with metabolic disorders such as diabetes and obesity, Wilms tumor of the kidney, adrenocortical tumors, and various other forms of cancer [27–31]. The cause of *H19/Igf2* epimutations remains unknown, however, studies suggest the genetic mutations may act by disrupting epigenetic stability at the locus [22–26]. While these mutations alone in the mouse do not perturb epigenetic status [20,21], this study will investigate whether the presence of these mutations increase sensitivity to VIN-induced epimutation.

2. Materials and methods

2.1. Animals – genetic line descriptions, sources, and housing

Animal handling was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* under the corresponding animal use protocol at the University of North Carolina at Chapel Hill and housed at the David H. Murdock Institute Vivarium in Kannapolis, NC. Five mouse lines were used in the study including: (1) wild-type fully inbred C57BL/6 (B6) mice; (2) wild-type C7 mice homozygous for CAST/EiJ across chromosome 7 and mixed CAST/EiJ-B6 genetic background [32]; (3) mutant 8nrCG mice [20] carrying targeted mutation of 8 base pairs at 8 CpGs at the *H19/Igf2* locus; (4) mutant ΔIVS mice [20] carrying a targeted deletion of 0.9 kb at the *H19/Igf2* imprinting control region; and (5) mutant Δ2,3 mice [21] carrying a targeted deletion of 1.3 kb at the *H19/Igf2* imprinting control region. All mutant lines were previously backcrossed 6–10 generations into B6 to result in mostly (>98%) B6 genetic background as confirmed by genotyping. B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used directly (study 1) or purchased separately and bred in-house to generate animals for study 2. B6-Cast7 (C7), 8nrCG, Δ2,3, and ΔIVS were derived in Dr. Marisa Bartolomei's lab at the University of Pennsylvania [32,20,21], then transferred and bred in-house at the David H. Murdoch Institute Vivarium in Kannapolis to generate animals for study 1 and study 2. Sterilized water and rodent chow were fed *ad libitum*. All mice were euthanized in accordance with current recommendations by the American Veterinary Medical Association (AVMA) guidelines. All tissues were collected and flash frozen in liquid nitrogen following euthanasia.

2.2. Breeding and treatments (see Fig. 1 for specific cross descriptions and timelines)

Supplemental Table 1 lists the number of pups, males, females, litters (dams), and average number of males per litter assessed in each experimental group. For generating G_1 animals, G_0 sires and dams (virgin females) were group housed after weaning/purchasing and set up to breed at approximately 8–10 weeks of age. Use of G_0 C7 males allowed for consistency in paternal contribution between lineages and also allowed for allelic expression or methylation analyses should the data have shown significant changes in the latter. G_0 dams were bred to stud males (8–24 weeks of age) and checked for vaginal plugs every morning. The first morning of a successful plug was considered GD1 and plugged females were immediately separated from stud males and placed into treatment cages. Group housed females were separated before parturition to enable identification of each litter separately. G_1 pups were counted on the morning of birth to measure litter size, sexed at PND5 to measure the number of males and females, weaned at PND28, and euthanized at PND84. For deriving the second generation of animals (G_2), 10 wk old G_1 males and females treated *in utero* were intercrossed (avoiding brother sister mating) or simultaneously outcrossed to untreated inbred B6 females. G_2 pups were counted on the morning of birth, sexed at PND5, and euthanized at PND5.

All G_0 dams were placed on a low-phytoestrogen purified diet (Teklad, TD.95092) one-week prior to mating and throughout breeding and gestation until PND5 to standardize nutrient intake and minimize potential xenoestrogenic properties of soy based diet [33]. Pregnant females were group-housed based on treatment group and plug date and treated daily with either VIN (VIN) (analytical grade >99% purity, Chem Service Inc, PA), M2 (analytical grade >99% purity, Cayman Chemical, MI), or pure corn oil (CON) (Sigma) by intraperitoneal injection. VIN and M2 were dissolved in corn oil at room temperature, prepared fresh weekly, and injected at a dose of 50 milligrams/kilogram of body weight/day (mg/kg/d) at a volume of 5 ul per gram of body weight. To reduce potential of lineage effects confounding the treatment effects, we avoided placing sibling females in the same treatment group and stud males were used across treatment groups. Mice were treated daily from GD9.5 to GD18.5. At PND5 for G_1 pups, dams and pups and were transferred to standard rodent chow (Teklad 8604) where they remained throughout subsequent breeding until the end of the study.

We performed two separate rounds of study. For both rounds of study, all methods of treatment and breeding schemes are maintained except where described (Fig. 1). Study 1 compared the effects of VIN and M2 to CON between two genetic lineages (crossed dam × sire): B6xC7 and 8nrCGxC7. G0 dams for these crosses were either homozygous wild-type (B6) or homozygous mutant 8nrCG mice. Study 2 compared the effects of VIN to CON among 5 genetic crosses (dam × sire): B6xC7, 8nrCGxC7, C7x8nrCG, ΔIVSxC7, and Δ2,3xC7. G_0 dams for these crosses were either homozygous wild-type (B6 and C7) or heterozygous mutant 8nrCG, Δ2,3, ΔIVS mice. See Supplemental Table 1 for litter and pup numbers.