International Journal of Andrology, in press

Developmental effects of chemicals: neonatal estrogen induced vaginal changes and prenatal organotin induced adipogenesis in mice

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Abstract

Developmental estrogen exposure induces persistent proliferation of vaginal epithelial cells in mice. This proliferation in the vagina of mice neonatally exposed to estrogens results from persistent phosphorylation of erbB2 and estrogen receptor α, sustained expression of EGF-like growth factors and phosphorylation of JNK1, IGF-I receptor, and Akt. The ubiquitous environmental contaminant, tributyltin chloride (TBT) is well known to induce the development of male sex characteristics (imposex) in gastropods. We recently found that TBT and its congeners induce the differentiation of adipocytes *in vitro* and increase adipose mass *in vivo* in vertebrates. TBT is a nanomolar affinity ligand for retinoid X receptor (RXR) in the rock shell and for both the RXRα and the peroxisome proliferator activated receptor γ (PPARγ) in the amphibian (*Xenopus laevis*), mouse, and human. TBT promotes adipogenesis in the murine 3T3-L1 cell model and perturbs key regulators of adipogenesis and lipogenic pathways *in vivo*, primarily through activation of RXRα and PPARγ. Moreover, *in utero* exposure to TBT leads to strikingly elevated lipid accumulation in adipose depots, liver, and testis of neonate mice and results in increased adipose mass in adults. In *X. laevis*, ectopic adipocytes form in and around gonadal tissues following organotin, RXRα or PPARγ ligand exposure. TBT represents the first example of an environmental endocrine disrupter that promotes adverse effects from gastropods to mammals. Prenatal (TBT) and early postnatal exposures (estrogens) stand as strong examples of endocrine disrupting compounds that permanently alter developmental programming. This emerging paradigm, the fetal origin of disease, is a new framework for considering the

effects of endocrine disrupters on human and animal health.

Key words: perinatal DES exposure, organotins, RXRα, PPARγ, adipogenesis, obesity

Introduction

Environmental endocrine-disrupting chemicals (EDCs) interact with steroid, arylhydrocarbon, retinoid and other nuclear receptors to regulate gene expression. Therefore, receptor-based functional assays are used in screening assays to detect biological activity of environmental chemicals (1). Estrogenic chemicals have been detected in aquatic environments worldwide and endocrine disruption in wild animals and humans exposed to EDCs during embryonic development has been summarized (2). Potentially new mechanisms for EDC action, such as proteasome-mediated degradation of receptors and their coregulators, genome-wide effects on DNA methylation status and modulation of lipid metabolism and adipogenesis, have recently been postulated (3).

The perinatal mouse model has been used to demonstrate the long-term effects of early exposure to estrogenic chemicals exposure on the female reproductive tract (4-6). Neonatal treatment of female mice with estradiol (E2), the synthetic estrogen, diethylstilbestrol (DES), or the xenoestrogen, bisphenol A (BPA) induces abnormalities in the reproductive tracts, hypothalamo-hypophysial-ovarian axis, immune system, and skeletal and muscular tissues. The growth response of perinatally DES-exposed reproductive organs to estrogen is altered, as are levels of estrogen receptors (ER), epidermal growth factor receptor (EGFR), prolactin receptor, oncogenes and *Hox* genes (7,8).

Organotins are a diverse group of widely distributed environmental pollutants. Tributyltin chloride (TBT) and bis(triphenyltin) oxide (TPT), cause multiple adverse effects on the invertebrate and vertebrate endocrine systems. Organotins were introduced as antifouling agents in marine shipping paints in the 1960s. Although the use of TBT in antifouling paints has been restricted, organotins continue to occur as contaminants in dietary sources, such as fish and shellfish, and through their use as pesticides and fungicides on food crops (9). Humans are also exposed to organotins through their use in wood treatments, industrial water systems, and textiles. Mono- and di-organotins are used as stabilizers in the manufacture of polyvinyl chloride plastics, which introduces the potential for transfer by contact with drinking water and foods.

TBT-induced imposex (the superimposition of male sex characteristics on female gastropod mollusks) represents one of the most clear-cut examples of environmental endocrine disruption because effects occur at the doses to which organisms are exposed

(reviewed in 10). Imposex impairs reproduction in severely affected animals, resulting in significant population declines. TBT exposure also leads to masculinization of two fish species but TBT exposure has only been shown to result in slight effects on the mammalian reproductive tracts; no reports of altered sex ratios have been forthcoming (see 10). Hepatic-, neuro- and immunotoxicity were reported to be the major effects of organotin exposure in mammals; immunotoxicity is the primary regulatory endpoint for TBT exposure (11). Current understanding of how organotins disrupt the endocrine system is based on the ability of organotins to modulate the expression or activity of steroid regulatory enzymes and via less specific toxic effects secondary to mitochondrial damage (12-14). These data do not permit one to draw firm conclusions regarding whether organotins function primarily as protein and enzyme inhibitors *in vivo*, or instead regulate gene expression in a more direct manner.

This review examines the emerging paradigm in which prenatal, or early postnatal exposure to environmental chemicals leads to permanent phenotypic changes, irrespective of subsequent exposure. This “fetal origins of disease” model provides important insights into the etiology of chronic diseases. It has considerable support from epidemiological studies, but much less in terms of molecular details. We discuss two model systems for which molecular mechanisms are becoming clear: the induction of persistent vaginal proliferation in neonatally estrogen exposed mouse vagina and the role of environmental obesogens in adipogenesis, focusing on changes in gene expression that may be responsible for the effects observed.

Analysis of estrogen responsive genes in mouse organs

To understand the mechanisms through which estrogenic chemicals act on mouse reproductive organs, information about the temporal and spatial expression patterns of estrogen-responsive genes is essential. A large number of genes affected by estrogen treatment were identified in tissues of wild-type, but not ERα knockout mice (15-17). For most of these genes, expression was induced by E2 in a dose-dependent manner. Characteristic gene expression patterns were observed for each xenoestrogen; these patterns were distinct from that of E2 (18-21). Estrogenic chemicals and dioxin have distinct effects on the liver (18, 20). Therefore, possible tissue specific effects should be considered when elucidating the distinct effects of various EDCs.

Persistent vaginal changes induced by perinatal estrogen exposure

Developmental exposure to DES induces carcinogenesis in human and laboratory animals. In mice, neonatal DES treatment induces persistent proliferation and

keratinization of the vaginal epithelium resulting in cancerous lesions later in life. Persistent phosphorylation of erbB2 and ERα, and sustained expression of EGF-like growth factors and phosphorylation of JNK1, IGF-I receptor and Akt, occurs in the vagina of neonatally estrogen exposed mice (22). DNA microarray analysis of the neonatally DES-exposed mouse vagina showed that expression patterns of genes related to cellular signaling were altered. We also found high expression of interleukin-1-related genes accompanied by phosphorylation of JNK1. Expression of IGF-1 and its binding proteins was modulated and led to phosphorylation of IGF-1 receptor and Akt, which is one of the downstream factors of IGF-1 signaling (23). In order to understand the molecular basis of the critical period of DES-induced vaginal changes, patterns of gene expression induced by DES were analyzed using DNA microarrays. The number of DES-induced genes during the critical period, PND 0, was smaller than those found after the critical period (24). *In utero* DES exposure induced changes in expression of genes such as DKK2, Nkd2 and sFRP1 as well as changes in genes of the Wnt, and Eph families in the female reproductive tracts. These genes could be the basis for various reproductive tract abnormalities following exposure to DES (25). Analysis of methylation status of genes showing altered expression in the mouse vagina is underway.

Adipogenesis stimulation by organotins in vertebrates

It is widely believed that high calorie diets, coupled with reduced physical activity are the major, if not the only cause of the rising worldwide incidence of obesity. The role of genetic factors is uncertain, but it is difficult to imagine a scenario in which individual genetic variations could be responsible for the rapid worldwide increase in obesity. A more reasonable idea is that interaction with the modern environment exposes underlying genetic differences that affect obesity. The Barker hypothesis postulates that *in utero* fetal nutritional status is a potential risk factor for obesity and related diseases (reviewed in 26). Developmental programming would change the setpoint for individual responses to diet, exercise, and environment. One such developmental programming event could be exposure to xenobiotic chemicals which are becoming increasingly prevalent in the environment. Our environmental “obesogen” model predicted the existence of xenobiotic chemicals that inappropriately regulate lipid metabolism and adipogenesis to promote obesity (26, 27).

Recent work has shown that aromatase mRNA levels are down-regulated in human ovarian granulosa cells by treatment with organotins or ligands for the nuclear hormone receptors, RXR or peroxisome proliferator activated receptor gamma (PPARγ) (26-29).

The gastropod *Thais clavigera* RXR homolog is responsive to 9-*cis* RA and TBT, and 9-*cis* RA can also induce imposex (reviewed in 10). We found the similar effects of TBT and RXR/PPARγ ligands on mammalian aromatase mRNA expression intriguing and hypothesized that TBT might exert some of its biological effects as a transcriptional regulation of gene expression. Accordingly, we tested the ability of TBT to activate a panel of nuclear receptors.

The results showed that TBT is a high-affinity ligand for RXRα and PPARγ and induces adipogenesis in cultured cells and in animal models (26-28). Several organotins activated both receptors. Remarkably, organotins of relatively diverse 3-D structures (e.g., TBT and TPT) are efficient activators and ligands for these receptors. The strong binding affinity of organotins for the receptors, taken together with the ability of organotins to compete with high-affinity RXR and PPAR ligands for receptor binding, suggest that organotins are bona fide ligands for both RXRs and PPARγ. TBT activates both receptors at nanomolar concentrations, far below the micromolar levels at which other mechanisms of toxicity become apparent.

The RXRα-PPARγ heterodimer is a master regulator of adipocyte differentiation and lipid storage; it also directly regulates lipid metabolism. Activation of PPARγ by TBT or specific ligands promotes the expression of genes that increase fatty acid storage and inhibits expression of genes that induce lipolysis (27, 28). Prenatal exposure to TBT led to persistent changes in the exposed animals such that they became 15% heavier later in life (27, 28). This is consistent with the increased body fat mass observed in patients treated with anti-diabetic thiazolidinediones that act through PPARγ to increase triglyceride storage in adipocytes and increase adipocyte numbers (30). The ability of thiazolidinediones to increase adipocyte number and fat mass suggests that TBT exposure could affect obesity at any time in life. We are currently investigating whether TBT treatment induces increased fat mass by increasing the number of adipocyte precursors, enhancing adipocyte differentiation from the same number of precursors, promoting proliferation of mesenchymal stem cells that become fat cells, or some combination of these.

Conclusion

Developmental estrogen exposure induces persistent proliferation of mouse vaginal epithelium, which is accompanied by persistent phosphorylation of erbB2 and ERα, sustained expression of EGF-like growth factors, and phosphorylation of JNK1, IGF-I receptor and Akt. Organotins such as TBT and TPT act as RXR activators, resulting in the development of imposex in the rock shell. They also act as chemical stressors or

“obesogens” that activate RXR and PPARγ signaling to promote long term changes in adipocyte number and/or lipid homeostasis following developmental or chronic lifetime exposure in vertebrates.

Acknowledgements

Partly supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Ministry of the Environment of Japan; the Ministry of Health Labour and Welfare of Japan (to T.I.), and grants from the U.S. Environmental Protection Agency (STAR R830686) and National Institutes of Health (GM-60572) (to B.B.).

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