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Review

Perinatal exposure to environmental estrogens and the development of obesity

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Dietary substances and xenobiotic compounds with hormone-like activity can disrupt the programming of endocrine signaling pathways that are established during perinatal differentiation. The consequences of this disruption may not be apparent until later in life but increasing evidence implicates developmental exposure to environmental hormone-mimics with a growing list of adverse health effects including reproductive problems and increased cancer risks. Obesity has recently been proposed to be yet another adverse health consequence of exposure to endocrine disrupting substances during development. There is a renewed focus on identifying contributions of environmental factors to the development of obesity since it is reaching worldwide epidemic proportions, and this disease has the potential to overwhelm healthcare systems with associated illnesses such as diabetes and cardiovascular disease. Here, we review the literature that proposes an association of perinatal exposure to endocrine disrupting chemicals, in particular those with estrogenic activity, with the development of obesity later in life. We further describe an animal model of developmental exposure to diethylstilbestrol (DES) to study mechanisms involved in programming for obesity. Our experimental data support the idea that adipocytes and the mechanisms involved in weight homeostasis are novel targets of abnormal programming of environmental estrogens, some of which are found in our foods as naturally occurring substances or inadvertently as contaminants.

Keywords: Adipocytes / Epigenetic / Leptin / Metabolic disease / Obesogens

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1 Introduction

Obesity is defined as excessive body fat (>25% men; >30% women). It is fast becoming a significant human health crisis and is receiving worldwide attention [1]. The prevalence of obesity has risen dramatically in wealthy industrialized countries over the last 2–3 decades, but it is also on the rise in poorer nations. The World Health Organization (WHO) has declared excessive weight as one of the top ten health risks in the world, and has estimated that the number of overweight people in the world is now greater than the number of undernourished. In the United States, obesity has reached epidemic proportions with more than 20% of adults being defined as clinically obese and an additional 30% defined as overweight (U.S. Department of Health and Human Services 2005; The Surgeon General's call for action to prevent and decrease overweight and obesity available at http://www.surgeongeneral.gov/topics/obesity). Obesity and overweight have been documented to seriously affect human health, and to impact the risks and prognosis for a host of serious medical complications including Type 2 diabetes, hyperinsulinemia, insulin resistance, coronary heart disease, high blood pressure, stroke, gout, liver disease, asthma and pulmonary problems, gall bladder disease, kidney disease, reproductive problems, osteoarthritis, and some forms of cancer [2–4]. Certainly obesity is a significant health risk for adults but it is a far more serious problem for children with the incidence of Type 2 diabetes, usually considered an adult onset disease, dramatically increasing in children and adolescents along with the rise in obesity. Since overweight children have a greater chance of becoming overweight adults, their prospect for a healthy future is not promising.

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Abbreviation: DES, diethylstilbestrol

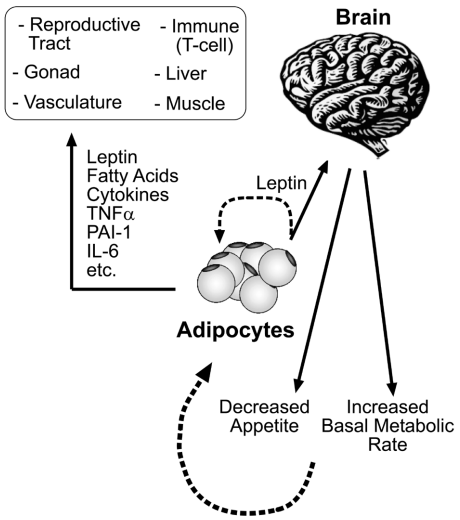

Figure 1. Adipocytes as an endocrine organ. The schematic shows the complex interactions of adipocytes with other tissues of the body.

Obesity, like many other chronic health problems, is known to be caused by a complex interaction between genetic, behavioral, and environmental factors. Commonly held causes of obesity are overeating and a sedentary lifestyle imposed on a background of genetic predisposition for the disease. Although much research has focused on these factors including the need to incorporate healthy foods in our diets and more exercise into our lifestyle, the exact etiology of obesity is unclear and it remains puzzling why some people are more successful in dieting and losing weight than others. It is clear, however, that obesity is very difficult to treat, therefore, prevention is essential.

Until the 1990s, adipocytes were considered to be just storage depots for excess metabolic fuel. However, the discovery of leptin as an adipocyte-derived hormone that communicated energy reserve information from adipocytes to other organs of the body including the central nervous system, lead to a new appreciation that "fat storage cells" are actually endocrine organs [2]. Mounting evidence has shown that adipocytes secrete a large number of cytokines and growth factors that play significant roles in growth and differentiation as well as in the feedback of information to other endocrine organs. Figure 1 schematically demonstrates the emerging concept of adipocytes as endocrine organs; the diagram continues to evolve and increase in complexity as we learn more and more about adipogenesis and mechanisms of its control. Considering the endocrine function of adipocytes and knowing that exposure to endocrine disrupting chemicals during critical periods of development can alter endocrine signaling pathways, we questioned if exposure to chemicals in the environment was related to obesity or any of its associated diseases.

Interestingly, a novel hypothesis has been proposed that *in utero* and/or early developmental exposures to environmental chemicals play a role in the development of obesity later in life. This idea parallels the experimental models for the action of environmental endocrine disrupting chemicals that are known to adversely affect aspects of reproductive endocrinology and health [5, 6]. A review published in 2002 postulated a role for chemical toxins in the etiology of obesity and summarized data showing that the current obesity epidemic coincides with the marked increase in use of industrial chemicals in the environment over the past 40 years [7]. In this article, data were presented that showed the current obesity epidemic cannot be explained solely by alterations in food intake and/or decrease in exercise. The author cited numerous studies where chemicals including pesticides, organophosphates, polychlorinated biphenyls, polybrominated biphenyls, phthalates, bisphenol A, heavy metals, and solvents caused weight gain possibly by interfering with weight homeostasis such as alterations in weight-controlling hormones, altered sensitivity to neurotransmitters, or altered activity of the sympathetic nervous system [7]. Most of the experimental studies cited in the review were conducted to detect toxicity of a specific chemical as determined by decreased weight; thus, a chemical was concluded to be nontoxic at certain levels if it caused no weight loss. Determination of weight gain was not an endpoint determined in the original design of these studies and, if noted, was considered to be a sign of "no toxicity" rather than an adverse effect. It is interesting that in a few cited studies, the chemicals were actually designed to have growth-promoting properties such as with diethylstilbestrol (DES) which was widely used by the livestock industry [8].

Since the Baille-Hamilton review [7], an increasing number of studies have been specifically designed to address the effects of environmental chemical exposure on weight gain and loss. These studies have shown that exposure to chemicals during critical periods of differentiation, at low environmentally relevant doses, alters developmental programming which can result in obesity. In fact, recent studies have shown that xenobiotic chemicals such as tributyl tin, found in PVC plastics and some fungicides, can disrupt normal development and homeostatic controls over adipogenesis and energy balance, resulting in obesity [9, 10]; these chemicals were termed environmental "obesogens" [10].

In vitro studies using 3T3-L1 cells (mouse fibroblasts that can differentiate into adipocytes) also suggest a link between environmental chemicals including tributyl tin [11], bisphenol A [12 – 14], nonylphenol [15], and genistein [16] in the development of overweight and obesity. Studies using pancreatic cells in culture also suggest that environmentally relevant doses of bisphenol A and DES can affect the normal physiology of the endocrine pancreas by altering the regulation of glucose and lipid metabolism [17].

The role of environmental chemicals in the development of overweight and obesity is an interesting area of emerging research which requires more knowledge especially in the identification of obesogens, their possible numerous molec-

ular targets, and potential cellular mechanisms through which they might act. Thus, to determine if environmental chemicals with estrogenic activity are playing a role in the development of obesity and, further, study potential mechanisms involved, we used an experimental mouse model of perinatal DES exposure which was developed and characterized in our laboratory to study altered developmental programming of the reproductive tract which is well known to result in disease and dysfunction [18].

2 The developmental exposed DES animal model to study obesity

Research in our laboratory has focused on the effects of estrogenic compounds on development and differentiation for over 30 years. Our working premise has been that "the developing organism is extremely sensitive to perturbation by chemicals with estrogenic or endocrine disrupting activity, and that exposure to these chemicals during critical stages of differentiation may have permanent long lasting consequences, some of which may not be expressed or detected until later in life." Since DES is such a well-known example, we have used it as a model chemical to study environmental estrogens. DES is a synthetic estrogen that was widely used for numerous purposes including in the livestock industry as a growth promoter for cattle [8]. It was also prescribed from the 1940s through the 1970s for the prevention of threatened miscarriage. Approximately, 2–8 million pregnancies worldwide have been estimated to be exposed to DES. It is well recognized today that prenatal DES treatment results in a low incidence of neoplasia in the female offspring, but a high incidence of benign abnormalities in both the male and female offspring [19]. To study the mechanisms involved in DES toxicity, we developed an animal model using outbred CD-1 mice treated with DES by subcutaneous injections on days 9–16 of gestation (the period of major organogenesis in the mouse) [20] or days 1–5 of neonatal life [21] (a period of cellular differentiation of the reproductive tract, and a critical period of immune, behavioral, and adipocyte differentiation). The developmental DES animal model has been used to successfully duplicate, and in some cases, predict, many of the alterations (structural, function, cellular, and molecular) observed in similarly DES-exposed humans [18, 22].

Although our major focus has been on reproductive tract abnormalities and subfertility/infertility, we also examined the effects of DES on body weight. Treatment of female mice with DES on days 1–5 of neonatal life using a dose of 0.001 mg/day did not affect body weight during treatment but was associated with a significant increase in body weight as adults [23]. Further, data indicated that the increase in body weight in DES-exposed mice was associated with an increase in the percent of body fat. Using mouse densitometry (Lunar PIXIMUS, GE Healthcare, Waukesha, WI), a representative image was generated that showed the size difference between control and DES-exposed mice (Fig. 2) [23].

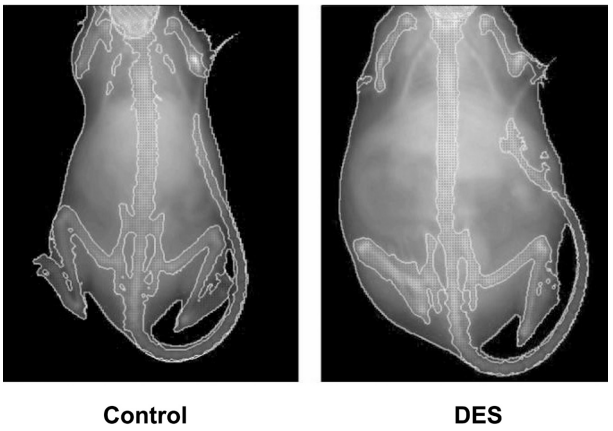

Figure 2. PIXI mus mouse densitometry. Neonatal exposure to DES (0.001 mg/kg) caused an increase in percent body fat compared to controls at 4 months of age. Figure is taken from ref. [23].

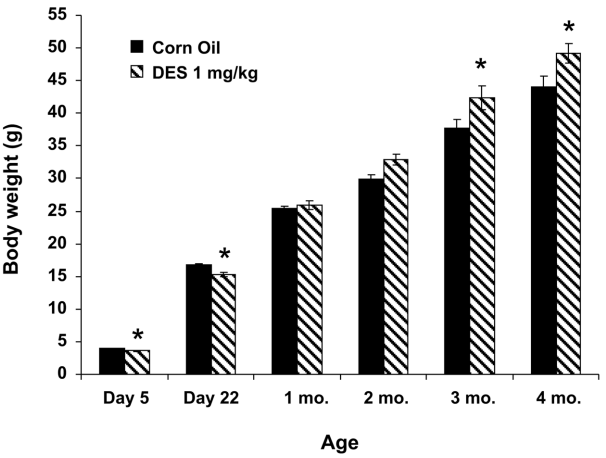

Figure 3. Body weight comparison of control and DES-treated mice during the time of treatment and continuing into adulthood. Mice were treated neonatally with DES 1 mg/kg or corn oil (control) on days 1–5 and body weight was taken at various ages. Numbers plotted are the mean body weight (g) ± S.E.M.

Unlike the lower dose of DES, the highest neonatal DES dose of 1 mg/day caused a significant decrease in body weight of female mice during treatment which was followed by a "catch up" period lasting until about 2 months of age and then finally resulted in a significant increase in body weight of DES treated mice as compared to controls [24]; Fig. 3 shows the body weight comparison of control and DES treated mice during the time of treatment and continuing into adulthood. The higher body weight in the DES treated mice was maintained throughout adulthood, however, by 18 months age, statistical differences in body

weight between treated and control females were difficult to determine due to increased individual animal variability within groups as they aged [24].

Examination of these DES 1 mg/day mice using mouse densitometry indicated that the increase in body weight in DES-exposed female mice was associated with an increase in the percentage of body fat and the mice appeared similar in size to the low dose DES mice shown in Fig. 2. Measurements obtained from densitometry of 6-month-old DES 1 mg/day mice showed an increase in the estimated body weight (45.0 ± 2.5 g in DES *vs.* 37.9 ± 2.5 g in Control), estimated fat weight (18.4 ± 1.8* g in DES *vs.* 11.5 ± 1.3 g in Control), and percent fat (40.8 ± 1.4* in DES *vs.* 29.9 ± 1.8 in Control), and a decrease in lean muscle mass percent (59.2 ± 1.8* in DES *vs.* 70.1 ± 1.8 in Control) [24]. Since the densitometry images suggested the DES treated mice had excessive abdominal fat which has been previously reported to be associated with cardiovascular disease and diabetes [25], the weights of various fat pads were measured to determine if specific fat pads were affected by DES treatment or whether it was a generalized effect throughout the mouse. At 6 – 8 months of age, all fat pads weighed more in the DES treated mice as compared to controls, but, only the inguinal (0.207 ± 0.041* g in DES *vs.* 0.108 ± 0.015 g in Controls) and retroperitoneal fat pads (0.555 ± 0.080* g *vs.* 0.291 ± 0.029 g in Controls) showed statistical significance [26]. Whether there is a statistical significance in the parametrial and gonadal fat pads that can be shown at older ages continues to be studied. Brown fat weights were not different between DES and controls [26].

A recent study supports a role of developmental genes in the origins of obesity and body fat distribution in mice and humans [26]. Therefore, exposure to environmental chemicals with hormonal activity may be altering gene expression involved in programming adipocytes. Several genes have been implicated in altering adipocyte distribution and function such as *Hoxa5*, *Gpc4*, and *Tbx15* and fat cell distribution such as *Thbd*, *Nr2f1*, and *Sfrp2*. We investigated changes in gene expression by microarray analysis in uterine samples from DES treated mice compared to controls at 19 days of age. In these samples, genes involved in adipocyte distribution were not altered in the uterus following neonatal DES exposure, however, genes involved in fat distribution were. *Thbd* and *Nr2f1* were significantly downregulated and *Sfrp2* was significantly upregulated in DES treated uteri compared to controls (data taken from study published by Newbold *et al.* [27]). These findings support the idea that environmental estrogens may play a role in regulating the expression of obesity related genes in development, however, additional studies are needed.

Serum profiles, determined at 2 and 6 months of age, showed that although the DES 1 mg/day treated female mice were similar in weight to controls at 2 months, they already had elevated levels of leptin, adiponectin, IL-6, insulin and triglycerides before overweight and obesity were detected suggesting these may be important early markers of subsequent adult disease [24]. All serum markers remained significantly elevated at 6 months of age except triglycerides [24] which we are further evaluating.

Since, the balance of activity levels and food intake are known contributors to obesity, we measured activity in DES 1 mg/day and control mice at 2 months of age before a difference in body weight could be detected. Individual mice were placed in an Opto-Max motor activity chamber (Columbus Instruments, Columbus, OH) and their ambulatory activity measured at 1 min intervals for a total of 20 min each. In both DES and control groups, activity levels dropped from the first 5 to 15 min intervals as the mice acclimated to their environment. Overall, there was no statistical difference in this parameter between the two groups although the DES group showed less movement at 5 min than controls. This difference was not enough to explain the enhanced weight gain in the DES mice as they aged [24]. However, other measures of activity are necessary before lack of activity can be ruled out as a contributing factor for the development of obesity in these mice.

Feed consumption was also measured over a 2-wk period. Control and DES-treated mice were individually housed and provided a preweighed amount of NIH-31 laboratory chow which has been reported to contain low levels of the phytoestrogens genistein and daidzein [28]. At the end of each wk, the remaining chow was measured and the total amount was subtracted from the starting amount to determine total feed consumed for each mouse *per* wk. Although the DES-treated mice ate more than controls over the course of the experiment (~3 g more), the amounts were not statistically different [24]. Taken into account, the marginal decrease in activity and the increase in food intake in DES treated mice as compared to control mice; it is unlikely these two measurements can solely explain the development of obesity in the DES treated mice.

The effects of long-term exposure to an enriched environment (access to a running wheel) on weight gain over time between DES treated and control mice were measured. At weaning, DES treated and control mice were each divided into two groups, one with access to a running wheel and the other with no access (n = 8 groups). Body weights were taken on the day of weaning and monthly thereafter until 6 months of age (Fig. 4). Control mice showed little reduction in the amount of weight gained following access to the running wheel at any time point examined. Interestingly, DES treated mice showed a decrease in weight gain when given access to a running wheel compared to no wheel (unpublished work). This could be due to differences in metabolism or differences in the use of the wheel or some other mechanism. The reason for the apparent protection against DES induced weight gain is not known but this is an area of research that could provide insight into the relationship between environmental influence and obesity.

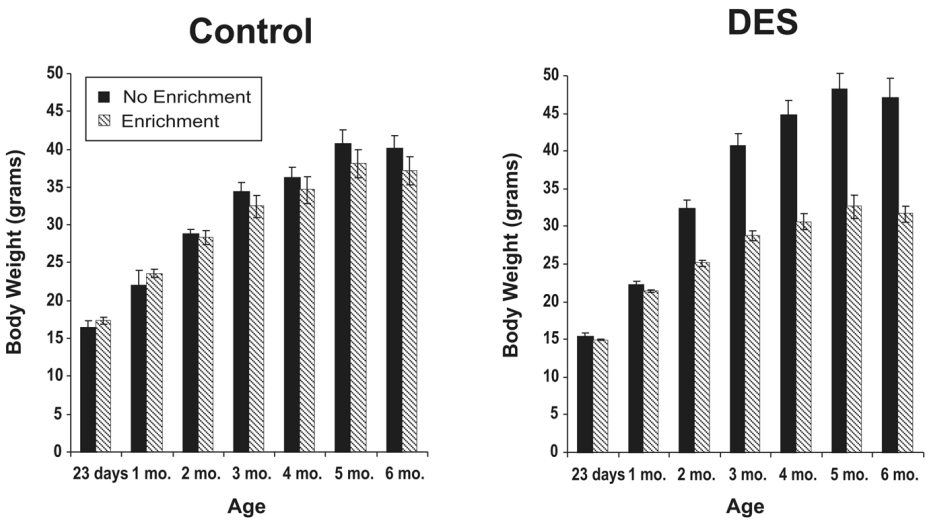

Figure 4. Body weight comparison of control and DES-treated mice under enrichment conditions *vs.* no enrichment. Mice were treated neonatally with DES 1,000 μg/kg or corn oil (control) on days 1 – 5 and weaned at 23 days of age to 4 females *per* cage. Mice were either provided enrichment (running wheel) or not ($n = 8$ mice *per* group). Numbers plotted are the mean body weight (g) ± SEM.

Glucose levels were also measured in DES and control mice at 2 months of age before obesity developed [24]. Following fasting for 18 h, serum was collected to provide the 0 time point. Mice were then weighed and challenged with glucose solution (2 g/kg body weight) by intraperitoneal injection. Blood was analyzed at 20, 40, 60, 80, 100, 120, 140, 160, and 180 min following glucose challenge. The results indicated that there was a wide range of response. However, two mice out of eight in the DES treated group had significantly higher glucose levels at 20 min following challenge than controls and other DES mice; these two mice also showed a slower clearance rate of glucose from the blood since higher levels were seen throughout the experiment; their glucose levels did fall by 160 min [24]. Altered glucose levels were observed in these two mice before they developed excessive weight. Glucose measurements are being determined in 6-month-old mice to determine if more mice are affected, and if higher and sustained levels of glucose are seen. However, to date these data suggest that overweight and obesity observed in perinatal DES treated mice will be associated with adverse health consequences such as the development of diabetes in mice as it does in humans. In fact, earlier studies from our laboratory have shown a high prevalence of islet cell hyperplasia in the pancreas of DES treated mice supporting the idea that these mice have abnormal glucose metabolism (unpublished work).

3 Summary and conclusions

Our data support the idea that brief exposure early in life to environmental endocrine disrupting chemicals, especially those with estrogenic activity like DES, increases body weight as the mice age. Whether our results can be extrapolated to health hazards in humans as the reproductive abnormalities from the DES mouse model did, remain to be determined but it provides a fruitful area of future research. In addition, the use of this animal model to study "obesogens" and mechanisms involved in altered weight homeostasis (direct and/or endocrine feedback loops, *i.e.*, ghrelin, leptin, *etc*.) by environmental endocrine disrupting chemicals is an important basic research area that may be addressed by using this model. We can no longer simply assume that overweight and obesity are just personal choices based on the foods we eat, but that complex events including exposure to environmental chemicals during development may be contributing to the obesity epidemic.

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