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Environmental Obesogens: Mechanisms and Controversies

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Abstract

Obesity is worldwide pandemic in adults as well as children and adds greatly to healthcare costs through its association with type 2 diabetes, metabolic syndrome, cardiovascular disease and cancers. The prevailing medical view of obesity is that it results from a simple imbalance between caloric intake and energy expenditure. However, numerous other factors are important in the etiology of obesity. The Obesogen Hypothesis proposes that environmental chemicals, termed "obesogens," promote obesity by acting to increase adipocyte commitment, differentiation and size, by altering metabolic setpoints or altering the hormonal regulation of appetite and satiety. Many obesogens are endocrine disrupting chemicals that interfere with normal endocrine regulation. Endocrine disrupting obesogens are abundant in our environment, used in everyday products from food packaging to fungicides. This review explores the evidence supporting the obesogen hypothesis, as well as the gaps in our knowledge that are currently preventing a complete understanding of the extent to which obesogens contribute to the obesity pandemic.

Keywords

adipogenesis; cancer; cardiovascular disease; developmental origins of health and disease; diabetes; DOHaD; EDC; endocrine disruptor; metabolic syndrome; obesity; obesogen; organotin; TBT; tributyltin; BPA; DEHP; phthalates; thermogenesis

INTRODUCTION

Obesity, defined as a BMI greater than 30 kg/m^2, is a global pandemic, affecting children and adults in developing as well as developed countries (1). An extensive systematic review of the literature (encompassing more than 68 million people) revealed that in 2015, at least 1.9 billion adults aged 18 and older were overweight and ~650 million of these were obese (2; 3). 107.7 million children worldwide were obese (2). In many countries, the rate of increase in obesity in children is greater than in adults (2) Overweight or obesity accounted for 4 million deaths globally and about 40% of these occurred in people who were overweight, but not obese. Excesss body weight accounted for 120 million disability-adjusted life years worldwide in 2015 (2) The prevalence of obesity in the United States more than doubled since the 1960s, from 13.4% to 35.7% in 2010 (4). Overall obesity

prevalence had increased to 37.7% by 2014 (5) and reached 39.8% by 2016 in the most recent analysis (6). Obesity disproportionately affects black (46.8%) and Hispanic (47.0%) populations with an even stronger effect on black (54.8%) and Hispanic (50.6%) women (6). More than one-third of youth in the United States were overweight or obese in 2012 (7); the prevalence of obesity in youth was 18.5% in 2016, with the highest incidence in the oldest group (20.6% in age 12–19 years) (6). Increased childhood obesity is strongly correlated with adult obesity and with increased diagnoses of type 2 diabetes in children and adolescents (8). Obesity and particularly increased visceral fat are recognized risk factors for metabolic syndrome (MetS), type 2 diabetes and other metabolic diseases (9). Obesity is also linked with the incidence of cancer; nearly 40% of all cancers are diagnosed in overweight or obese people in the U.S. (10). The additional cost of medical care attributable to obesity adds more than $200 billion annually to the U.S. health care budget (11). These excess costs derive from treating the many comorbidities of obesity such as cardiovascular disease, dyslipidemia, type 2 diabetes, liver disease, neurodegenerative disease, cancers and reproductive diseases (11–14).

CAUSES OF OBESITY

Disrupted energy balance does not account for the obesity pandemic

For many years, the clinical paradigm for obesity has been a simple function of energy intake versus energy expenditure (15). Other well-studied risk factors implicated in obesity include genetics (16; 17), smoking during pregnancy (18; 19), stress (20; 21), the microbiome (22–24), and timing of meal consumption (25; 26) to name a few. The most prevalent clinical management strategies are focused on managing diet and increasing exercise (27). This myopic approach continues despite numerous studies demonstrating that exercise is consistently linked with increased fat mass in the long term (28; 29). A recent analysis of data derived from the U.S. National Health and Nutrition Examination Study (NHANES, 1971–2008) showed that leisure time physical activity increased 47% in males and 120% in females between 1988 and 2006, yet for an equivalent amount of caloric intake, macronutrient intake and leisure time physical activity, average adult BMI was up to 2.3 kg/m^2 higher in 2006 than in 1988 (30). Thus, decreased physical activity and increased food consumption are not adequate to explain the increase in BMI between 1988 and 2006 (30). These results cast serious doubt on the sufficiency of the simplistic energy balance model of obesity and strongly implicate the importance of other risk factors in obesity (30).

Multifactorial Nature of Obesity

Under “normal” conditions the body typically autoregulates its weight; the brain senses and processes a variety of metabolic signals from several tissues and uses those data to control body weight. Body weight in adults is typically stable over a long time, balancing metabolic and hedonic mechanisms (31). A return to normal weight quickly follows illness-induced or diet-induced weight loss. Similarly, a weight gain of 5–10 lb as a result of dietary excess during a a holiday season, for example, is quickly lost when individuals return to their “normal” life. Thus the idea is emerging that body weight is regulated by a “body weight set point” (31). This idea is also supported by the observation that obesity is largely intractable once established. Multiple studies have reported that more than 83% of obese individuals

who lost substantial amounts of weight through rigorous adherence to a strict regimen of dietary restriction and exercise gained it back within a few years (32–34). It is difficult to understand why such a small fraction of people who have successfully remodeled their bodies can sustain this substantial weight loss without invoking the concept of a body weight point. How might such a set point be established?

Developmental origin of disease

Development is highly orchestrated and tightly controlled over time. The “Fetal Origins Hypothesis”, first proposed by David Barker posited that prenatal nutrition can play an important role in one’s susceptibility to metabolic diseases and cardiovascular disorders later in life (35; 36). In this view, the type and amount of prenatal nutrition experienced by a fetus led to adaptation to the intrauterine nutritional environment. If the fetus experienced malnutrition in utero, as revealed by being born small for gestational age, Barker believed that the fetus had become adapted to a nutritionally poor environment. If the actual environment were not nutrient poor, but rather nutritionally adequate or more, the child would be more susceptible to chronic diseases later in life, such as type 2 diabetes, high blood pressure, heart disease and obesity due to this mismatch. Environmental factors acting during critical periods of development led to subsequent programming events that could influence physiology permanently, increasing susceptibiltiy to diseases later in life (35; 36). Barker called this the “thrifty phenotype” that reflected early life programming as an altered ability to use and store calories later in life (36). Gluckman and Hanson recognized that such critical periods were not restricted to fetal development and extended the fetal origins model, renaming it as “The Developmental Origins of Health and Disease” or DOHaD paradigm (37–39). The overall result of fetal and early life programming events is physiological function within normal parameters but with underlying functional deficits that can increase susceptibility to disease and dysfunction, particularly when other stressors such as diet are superimposed later in life. Studies in animals and humans have borne out a connection between poor prenatal nutrition and increased risk for diseases such as obesity throughout the life course and even in subsequent generations (35; 40). Despite considerable study, there are very few concrete details on the molecular mechanisms that promote a thrifty phenotype, although, there are indications that leptin resistance (41) and epigenetic mechanisms are involved (42–44).

Obesity begins in the womb.

As noted above, the obesity pandemic has also reached the pediatric population, where 18.5% of American children aged 2 to 19 were obese (≥95th percentile on CDC growth charts), and 32% are overweight (≥85th percentile) (6; 7). It is also particularly alarming that obesity rates are also rising among children less than 2 years old (6). While an argument can be made that older children and adults might eat more and exercise less, it is difficult to argue that the typical infant now consumes more calories and exercises less than in previous generations. Therefore, it is more likely that the infant was born with more fat, and/or that something about the early pre- and poststnatal environment is different than in the past. Maternal nutrition,(35; 45) stress,(46) and insulin state(47) have all been linked to risk of obesity in offspring, supporting the contention that aspects of the prenatal and early life environment play important roles in determining whether an individual may be predisposed

to obesity and metaboli disease later in life (48). With respect to the environment, a provocative study found that animals living near humans in industrialized societies have also exhibited pronounced increases in obesity over the past several decades (49). These animals included domestic cats and dogs (which one might suppose eat too much and exercise too little), feral rats living in cities and, crucially, six different species of laboratory animal models. The laboratory models included four different species of primates living in National Primate Research Centers as well as laboratory rats and mice, all living in environments where their diets are strictly controlled (49). Therefore, rather than ascribing this increased obesity to energy balance, a more reasonable inference is that something has changed about the environment in which these animals live that is making them obese in parallel with humans.

Endocrine disrupting chemicals

The endocrine system is well-known to play an important role in energy balance, fat deposition and fat distribution in the body because many endocrine organs and hormones work together to regulate metabolism and body weight. Insulin and glucagon produced in the pancreas modulate glucose uptake and usage; ghrelin and cholecystokinin affect metabolism in the gastrointestinal tract; and glucagon, insulin, and fibroblast growth factor 21 (FGF21) act in the liver to control metabolism, hunger, and satiety. Sex hormones such as estradiol also contribute to energy metabolism and obesity by affecting food intake, body weight, fat distribution, and can alter the balance of glucose and insulin, lipogenesis, and lipolysis (reviewed in 48). The brain is an endocrine organ that both regulates metabolism and controls hedonic circuits that modulate food intake via reward mechanisms involving peptide hormones, neurotransmitters and growth factors (31; 50–52).

An important advance in our understanding of how the environment could influence health, particularly by influencing developmetnal programming, came from the recognition that chemicals could disrupt function of the endocrine system (reviewed in 53; 54). The Endocrine Society defines an endocrine disrupting chemical (EDC) as an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action” (55). This is distinct from the toxicological definition of an EDC which would add the rather vague requirement that such effects must be “adverse”. It is currently unclear how disrupting endocrine function might not be adverse, *per se*, as already widely recognized by endocrinologists and endocrine scientists (55).

The original definition of EDCs that was embodied in the Food Quality Protection Act of 1998 focused on estrogens, anti-androgens and thyroid disruptors but it is now recognized that any endocrine signaling system can be disrupted by exogenous chemicals that target different points in endocrine signaling pathways (54). For example, EDCs can affect how hormones are produced or destroyed, whether and how they might be transported through the blood and across cell membranes to their targets as well as modulating the molecular functions of the specific hormone receptors and their co-factors (reviewed in 53; 54). In addition to the 48 nuclear hormone receptors encoded in the human genome, all of which are potential targets for being disrupted, there are hundreds of peptide hormone signaling pathways as well as a large number of receptors for as yet unidentified hormones. Thus the

potential for disrupting the action of endocrine hormones is considerable. However, why should our consideration of potential cellular targets of environmental chemicals be limited to endocrine hormones? Any receptor-based cellular cellular signaling system can be disrupted by chemicals that act on the receptor. This leads to the concept of "signal toxicity" wherein chemicals can act on any type of receptor and at any part of a cellular signaling pathway to disrupt cellular function (56). A key feature of this model is rather than eliciting toxicity by damaging cellular components, chemicals that cause signal toxicity do so by interfering with signaling itself (usually by acting on the receptor). A general feature of such cellular signaling systems is that molecules capable of disrupting them are expected to act at low doses (comparable to that of the endogenous hormones) and not to exhibit threshold effects because the signaling systems are typically already active (57).

The obesogen hypothesis

In 2006, Blumberg and Grün proposed the existence of endocrine disrupting chemicals that could influence adipogenesis and obesity and be important, yet unsuspected players in the obesity epidemic. These "obesogens" are functionally defined as chemicals that promote obesity in humans or animals (58). The obesogen hypothesis further developed the DOHaD paradigm for obesity by proposing the existence of EDC obesogens that act during development to influence obesity later in life. Obesogens can act directly on cells to increase the commitment or differentiation of adipocytes from stem cells, by altering the number of adipocytes, by increasing storage of triglycerides into adipocytes, or by altering the rate of adipocyte birth versus destruction. Obesogens can also act indirectly by changing basal metabolic rate, by shifting energy balance to favor calorie storage, by modulating food intake and metabolism via effects on the brain, pancreas, adipose tissue, liver, gastrointestinal tract, brain and muscle (reviewed in 48; 59; 60). The effects of obesogens lead to an altered "set point" or sensitivity for developing obesity later in life (61; 62). This can exacerbate the effects of diet composition or caloric intake, making obesogens potential regulators of the body weight set point noted earlier. In this view, obesogens alone do not cause obesity in humans but can play an important "behind the scenes" role in weight gain due to developmental programming of the control of adipose tissue, food intake and metabolism. Thus, obesogenic EDCs work by affecting nuclear factors or other endocrine pathways during development in ways that lead to obesity later in life.

Mechanisms of obesogen action

The interaction of multiple factors across the lifespan and generations makes it difficult to determine precisely to what extent exposure to EDC obesogens contributes to the obesity pandemic in humans, despite its great importance and interest to scientists, the public and policy makers. To address this important question, one must examine the data that support or refute the the obesogen hypothesis and the extent to which these data are consistent with the importance of environmental obesogens in the etiology of obesity. This topic has been extensively reviewed in both human and animal studies (48; 59; 60; 63–68). The reader is directed to these reviews for a more complete listing of chemicals shown to be or purported to be obesogens. Evidence has accumulated linking EDCs to obesity, and obesogens have been detected in humans (69–73) and animals (74–77). Because these chemicals are pervasive in the environment, it is crucial to understand how they disrupt developmental

programming, predisposing individuals to obesity and related disorders. Although about 50 obesogens have been identified, (48) there is little mechanistic understanding of how most function. Below we briefly review the established mechanisms through which obesogens can act and examples of chemicals acting through each.

Obesogens and the programming of adipocyte development

One obesogen for which there is a considerable body of mechanistic evidence is tributyltin (TBT). TBT is widely used in industry and the related chemical, triphenyltin (TPT) continues to be used in agriculture. Human exposure occurs through dietary sources such as seafood and shellfish contaminated by TBT used in marine shipping applications or as fungicides for paper mills and industrial water systems. TPT use as a fungicide on high value food crops presents more opportunities for human exposure. TBT contaminates plastics (e.g., polyvinyl chloride) and house dust (78; 79). TBT is a nanomolar affinity ligand that activates the “master regulator” of adipogenesis, peroxisome proliferator activated receptor gamma (PPARγ) and its heterodimeric partner, 9-cis retinoic acid receptor (RXR), to promote adipogenesis and alter lipid homeostasis (76; 80; 81). Human and mouse mesenchymal stem cells (MSCs) and pre-adipocytes are induced to differentiate into adipocytes via a PPARγ-dependent pathway after exposure to TBT at environmentally-relevant (nanomolar) levels or via PPARγ agonists such as rosiglitazone (ROSI) (82–84). Adult mice exposed to TBT in utero displayed increased lipid accumulation in adipose depots, livers, and testis (76; 85). Although epidemiological studies of TBT levels and its associated effects are scant, TBT continues to be found in house dust (79) and in seafood, (86) and at least one study shows increased ponderal index in human infants with the highest prenatal TBT exposure (87).

In vivo experiments identified several other chemicals as known obesogens. Exposure to the fungicides triflumizole (83), tolylfluanid (88) or the plasticizer diethylhexyl phthalate (89) led to obesity later in life. Triflumizole was shown to act through PPARγ (83), whereas tolylfluanid is thought to act via the glucocorticoid receptor (90). It is not known which receptor diethylhexyl phthalate acts through, but it may be PPARγ.

Other potential obesogens have been identified that are known to act on preadipocytes or MSCs to promote adipogenic differentiation (91). TBT alters the genome-wide chromatin landscape by activating RXR to commit cells to the adipocyte lineage by decreasing the deposition of the repressive histone 3 lysine 27 trimethylation mark, leading to increased expression of genes favoring adipogenic commitment (92). Specific RXR activators (rexinoids) produce the same phenotype so it is expected that other chemicals that activate RXR will increase adipogenic commitment in MSCs. Many potential obesogens activate PPARγ such as the agricultural chemicals quinoxyfen, and spirodiclofen, whereas another, fludioxonil, activates RXR (91). The agrochemicals tebupirimfos, flusilazole, forchlorfenuron, acetamaprid and pymetrozine all induce 3T3-L1 preadipocytes to differentiate into adipocytes in culture by mechanisms other than activating PPARγ (91). Flame retardants, phthalates, plasticizers, parabens, alkylphenols and bisphenols can all differentiate 3T3-L1 cells to adipocytes in vitro (reviewed in 48). It remains to be shown

which of these chemicals are capable of inducing fat accumulation and obesity, in vivo, but one might reasonably expect that at least some will turn out to be bona fide obesogens.

Obesogens and unhealthy adipocytes

Considering how many chemicals increase the commitment or differentiation of adipocytes, it is reasonable to ask whether the adipocytes produced are normal and fully functional (93). White adipocytes are important in the maintenance of metabolic health by taking up glucose from the bloodstream in response to post-prandial insulin (94). White adipose tissue (WAT) also has endocrine functions, releasing adipokines such as adiponectin that are important for metabolic health (95). Adiponectin expression suppresses gluconeogenesis and stimulates β-oxidation of fatty acids in the liver and is inversely correlated with risk of type 2 diabetes (96). PPARγ activators are known to promote the development of healthy adipocytes (97; 98). Overall, functional adipocytes are characterized by their sensitivity to insulin, production of “healthy” adipokines such as adiponectin and being small, normoxic cells that promote an anti-inflammatory and anti-fibrotic local microenvironment.

Sargis and colleagues investigated functional differences in 3T3-L1 cells differentiated with TBT or the PPARγ activator troglitazone (88). They found that the TBT-induced adipocytes produced lower levels of adiponectin and CEBPα mRNA and protein, accompanied by reduced GLUT4 expression but normal glucose uptake and inferred that TBT had produced “unhealthy” adipocytes (88). Shoucri and colleagues treated differentiating MSCs with TBT, ROSI, or the rexinoid IRX4204 and found that while TBT- and 4204-treated cells accumulated essentially the same amount of fat as did ROSI-treated cells, the TBT- and 4204-induced adipocytes were abnormal. That is, they did not express GLUT4 to levels comparable to ROSI, attenuating glucose uptake, showed reduced expression of adiponectin and failed to down-regulate the expression of markers of inflammation and fibrosis (93). The TBT or 4204-treated cells also contained fewer mitochondria and did not respire at the rate achieved by ROSI-induced cells under stress (93). These cells were also impaired in their ability to produce thermogenic beige/brite fat as described in the next section. Overall, TBT or rexinoid differentiated adipocytes accumulated fat, but did not respond to normal signaling processes. This may be relevant to the in vivo phenotypes where F4 male animals derived from F0 TBT-treated dams stored excess triglycerides when dietary fat was elevated and failed to mobilized this fat under fasting conditions (61). It is an open and important question whether other environmental obesogens generate normal or dysfunctional WAT.

Obesogens and impaired thermogenesis

A dimension of adipocyte function that has advanced greatly in recent years is the discovery that thermogenic brown adipose tissue (BAT) persists in adult humans and that white adipose tissue (WAT) can be induced to produce thermogenic beige or brite fat (99; 100). This “browning” process produces thermogenic adipocytes that are characterized by abundant mitochondria. Expression of Uncoupling Protein 1 (UCP1) uncouples cellular respiration from ATP synthesis producing heat instead of ATP. Thermogenic adipose tissue has important therapeutic potential as a treatment for obesity and diabetes since it readily converts blood glucose and fats into heat (101). Two lines of evidence suggest that some obesogens can act, in part, by impairing the function or differentiation of thermogenic fat.

First, perinatal exposure to the insecticide dichlorodiphenyltrichloroethane (DDT) produces adult female animals that are intolerant to cold, exhibit diminshed energy expenditure and reduced core temperature (102). This phenotype of reduced BAT function resulted, in part from diminished expression of of a key regulator of BAT function, peroxisome proliferator-activated receptor γ coactivator 1α (Ppargc1α or PGC-1α) and iodothyronine deiodinase 2 (Dio2, which converts thyroxine, T4, to the more thermogenic triiodothyronine, T3). Second, Shoucri and colleagues recently found that production of beige/brite fat cells from MSCs was inhibited by TBT or rexinoids (93). These examples indicate that obesogens can influence obesity by impairing thermogenesis, in vivo. This is an intriguing area for future study.

Obesogens and altered metabolic setpoints

Developmental obesogen exposure can result in increased fat depot weight as well as adipocyte size and number in animal models. The situation in humans is likely to be more complex with a number of contributing (genetics, diet, microbiome, circadian rhythms, exercise, environmental stressors) and confounding (prescription drugs, multiple exposures, individual variations) factors. As noted earlier, it is clear that in humans it is easy to gain weight, hard to lose weight and even harder to maintain weight loss; all of these can be interpreted to indicate the existence of a body weight set point. If true, this indicates that while food intake and exercise are very important factors in weight control, obesogen exposure can alter the amount of food needed to increase weight or the amount of exercise needed to lose weight by altering this set point. It may require a “second hit” in order to gain weight from obesogen exposure.

Several examples suffice to indicate the presence of a body weight set point that has been altered by obesogen exposure. Newbold and colleagues showed that perinatal exposure to diethylstilbestrol led to weight gain at adulthood, but not earlier (103). Developmental exposure to BPA throughout gestation and lactation in pregnant mice resulted in adult female animals that showed decreased activity and energy expenditure, although, body weight and fat content were not assessed (104). Somm et al. showed that prenatal nicotine exposure altered the body weight set point in rats such that less high-fat food was required to gain weight than in controls (105). Chamorro-García and colleagues showed that ancestral exposure to TBT led F4 generation male mice to gain weight very rapidly when dietary fat was increased, failed to mobilize this fat during fasting, likely as the result of leptin resistance (61). Lastly, a recent human diet-induced weight loss study showed that while all subjects lost weight comparably when calories were restricted, those with the highest baseline plasma levels of perfluoroaklyl substances had lower resting metabolic rates and regained weight more rapidly (62). Taken together, these data strongly support the idea that obesogen exposure can alter body weight set points in multiple ways.

Transgenerational effects

An important recent advance in the understanding of the effects of obesogens came with the finding that when pregnant mice were treated with environmentally-relevant (nM) doses of TBT via their drinking water, effects were detected in the F1-F4 descendants of F0 mice exposed during pregnancy (85) or pregnancy and lactation (61). Unlike TBT, strong

activators of PPARγ such as ROSI could not elicit transgenerational effects on obesity (61; 85). TBT exposure led to a transgenerational “thrifty phenotype” in F4 male (but not female) mice. Males were resistant to fat loss during fasting, rapidly gained fat on a higher fat diet (HFD) and retained this fat when returned to a low fat diet (LFD). This was not simply a result of fat animals producing fat offspring because the phenotype is not manifested until diet challenge. The thrifty phenotype was associated with changes in chromatin structure, DNA methylation and overexpression of leptin and important metabolic genes in white adipose tissue (WAT) (61). Thus, it was proposed that transgenerational obesity resulted from maternal programming leading to a heritable susceptibility.

Heritable effects of other chemicals on obesity have been demonstrated. Plastic components BPA, diethylhexyl and dibutyl phthalates (106), the pesticide methoxychlor (107), a mixed hydrocarbon mixture (jet fuel JP-8) (108) and the pesticide, DDT (109) all induced transgenerational obesity in rats. Two major arguments have been raised against the concept of epigenetic transgenerational inheritance. First is that it presumes soma-to-germline transfer of heritable information, which is an anachronistic Lamarckian notion of inheritance and a violation of the Weismann barrier: that hereditary information moves only from the germline to the soma (110). However, recent advances in developmental cell biology of germline genomics challenge Weismann’s idea (111). Normal development of mammalian germline cells depends on hormonally regulated functions of somatic cells supporting their survival and differentiation. It is plausible that endocrine active substances may affect epigenetic reprogramming of germline cells. The second argument is that genome-wide epigenetic reprogramming in mammalian germline cells should erase epimutations from the preceding generation (110; 112; 113). While Skinner et al. reported that vinclozolin exposure induces transgenerational changes in DNA methylation through the F3 and F4 generations (109; 114; 115), Szabo et al. showed that vinclozolin-induced epimutations and alterations in gene expression are induced in the F1 generation, but were not conserved in F2 prospermatogonia (116). These contradictory results can be reconciled in a model proposed by Chamorro-García and colleagues (61). They posited that methylation at specific CpG sites does not need to be inherited but rather that changes in chromatin accessibility and/or organization convey the the transgenerational traits (61). In this view, altered genomic structure and accessibility to epigenetic writing enzymes are inherited and these lead to regional changes in DNA methylation that occur in the areas of altered structure, leading to differential gene expression (61). It remains to be seen how many other EDCs or obesogens lead to transgenerational inheritance of obesity or other phenotypes.

What can we know about the role of obesogens in obesity?

As noted above the etiology of obesity is multifactorial and cannot be linked specifically and wholly to one entity, including genetics or environmental chemicals. While dietary restriction and increased exercise continue to be the most prescribed remedy, the obesity pandemic continues unabated and is increasing worldwide (2). Despite the voluminous literature on obesogens and metabolism disrupting chemicals (reviewed in 48) a series of workshops aimed at identifying the best evidence for obesogen effects on obesity and diabetes identified shortcomings in the existing body of evidence that prevented a full and accurate analysis of the effects of many potential obesogens (14). What types of evidence

will be required to overcome these shortcomings and firmly establish obesogens as important players in the obesity pandemic? What will be the best practices for human and animal studies? Below we list some of these and then follow with general conclusions and suggestions for future studies.

General considerations

In addition to genetic background, stress, drugs, infections, other diseases and the microbiome the effects of exposure to environmental chemicals will be specifically influenced by:

- Timing of exposure. The *in utero* period may be the most sensitive, but our lack of understanding of all windows of sensitivity (which will differ by chemical) necessitates a developmental and lifetime approach.
- The window of sensitivity for an exposure to elicit a disease outcome may be longer than the time exposure was assessed. This will require multiple exposure assessments over longer times including preconception, early pregnancy and early childhood.
- Some effects will be due to functional changes, perhaps epigenetic in nature that will require sensitive and specific endpoints that might not become apparent until later in life, thus requiring a lifespan prospective approach.
- EDC and obesogen exposure typically occurs at low levels which vary significantly over time requiring multiple and sensitive measures of personal exposures.
- Multiple interacting exposures are typical, individual sensitivities and half-livesof chemicals will differ, requiring mixture and statistical approaches.
- Individuals will experience multiple “hits” over a lifetime, requiring a lifetime and statistical approach.
- Analysis of transgenerational effects will require biomarkers of ancestral exposure to identify who has been exposed.

Methodological considerations in epidemiological studies

Epidemiological studies suggest a link between chemical exposures and a variety of health outcomes later in life, including obesity (reviewed in 117). However, results are often inconsistent across studies which may be due to methodological challenges specific to epidemiology studies (reviewed in 118; 119). A key challenge is to accurately estimate aggregate exposures to chemicals which may occur via multiple routes, and have short half-lives resulting in concentrations that vary significantly over time. Potential misclassification of such exposures is particularly relevant for non-persistent chemicals; inter-individual variability both in exposures and in assessment must be addressed in the experimental design. Single spot urine or even a 24 hr urine samples may be problematic as they only assess exposure over a day or even a part of a day (reviewed in 63; 119). For example, at least ten and as many as 35 individual measurements may be needed to adequately assess individual BPA exposure (119; 120). It is also important to examine racial and ethnic

differences in exposures (121). Exposure of humans to many potential EDCs can be confounded because most existing cohorts and epidemiological studies were designed to measure the impact of a single chemical without accounting for the effects of mixtures (63). Epidemiology studies must develop the appropriate statistical models to determine the health outcomes associated with specific chemicals in the mixture and such models will need to deal with the fact that some EDCs act by the same mode and mechanism, while interfering with others. Particular care must also be taken to avoid sample contamination during collection, handling and storage, especially when assessing chemicals commonly found in laboratories and medical equipment (e.g., phthalates and bisphenol A).

It is also important that epidemiology studies assess the shape of the dose-response curve as some associations between exposures and health outcome may be non-monotonic (57). Such studies should also consider the importance of determining chemical concentrations during the entire sensitive window of exposure which may continue beyond in utero. Animal studies have revealed that obesogen effects on obesity are often sexually dimorphic, thus, epidemiological studies must consider both sexes and also overlapping endpoints that assess the function of multiple tissues and mechanisms.

In addition to all these challenges, it must be noted that in the absence of accidental or industrial exposures, epidemiological studies cannot definitively provide causal links between exposure and disease etiology. Thus the most scientifically valid approach to determining the importance of EDC exposures to obesity is to focus on animal studies where the study design can minimize effects of confounding variables and then assess links between exposures to such chemicals and the same disease outcomes in human cohorts.

Methodological Considerations in Animal Model Studies

While animal studies can provide important data, including causality, linking obesogen exposure and obesity important factors need to be addressed to assure a valid interpretable animal study. These include:

- Using a species and strain of animal known to be susceptible to the disease and that the disease mimics the human disease. A model useful for a cancer study may not be appropriate for an obesity study.
- Assess endpoints that are sensitive and linked to the disease, not just body weight.
- The use of positive controls is mandatory to ensure that the animal is responsive to the conditions of the study so that negative results can be taken as meaningful.
- Assess multiple overlapping sensitive endpoints that evaluate multiple tissues and functions in both sexes.
- Use a wide variety of concentrationsm, including some that are in the range of human exposures (often below the NOAEL) to determine whehther effects are relevant to humans and may be nonmonotonic.
- Whenever possible, measure circulating chemical and metabolites to compare doses to human blood levels.

- Use sufficient numbers of animals per group for statistical significance.
- Randomize the animals, blind the animal groups to the researchers and treat all groups in parallel to avoid batch effects.
- Use a diet containing protein, carbohydrates, phytoestrogens and lipid concentrations that will allow obesity to manifest in the species studied.
- When using a high-fat diet avoid the use of extremely high fat diets (e.g., 60% Kcal from fat) in favor of a diet that has modest levels of fat that can reveal effects while maintaining relevance to human biology.
- Dose during specific sensitive time periods appropriate to the purpose of the study, this could include a single or multiple exposures within a generation or across generations.
- Use a route of exposure that fits the purpose of the study and is most relevant to human exposures. When alternative routes of exposure are required for the experiment, attempt to measure blood levels achieved and compare to oral dosing.

When assessing the results of animal studies, it is also important to note that a positive result is more significant than a negative result since it is always possible to design an experiment that produces a negative outcome. It is also important to consider that it may not always be possible to perfectly replicate a study, at least in part due to local difference in microbiome composition. Therefore, even " replicate" studies may have different results. It is almost universally true that studies from diverse laboratories use different chemical doses, dosing periods, diets, housing conditions and strains of animals. Therefore, studies that do not report identical results do not necessarily disprove original positive or negative findings. It is also likely that different animal models may result in obesity via distinct mechanisms including effects on diverse tissues. Moreover, it is also important to note that there may be animal models, chemicals and doses of specific chemicals that will not cause obesity, *per se*, but rather alter the set point for gaining weight. In such case, a " second hit" may be needed, for example, a higher fat diet. Since this is likely to be the human situation, these studies may be extremely relevant.

We argue that when assessing the preponderance of evidence from animal studies relevant to a particular hypothesis all of the above considerations must be taken into account. It is insufficient to simply count the number of "positive" and "negative studies" and then infer whether a chemical can cause obesity.

Establishing a Causal Role for Obesogens in Obesity

The obesogen field is a bit more than a decade old. Over this first decade the small cadre of scientists working on the role of EDCs in obesity has grown and the work has shown that there are obesogens, defined in animal studies, and that these have the potential for effects in humans. Several international workshops have been held to discuss the potential role of EDCs in obesity and metabolic diasease (14; 48; 122; 123). There was wide agreement on the following points:

- The increase in obesity over the last 50+ years cannnot be accounted for by genetic factors alone.
- Obesity has genetic, epigenetic and an environmental components.
- The environmental component is multifactorial and includes environmental chemicals.
- Susceptibility to obesity is at least in part “programmed” during development by environmental factors broadly defined including stress, drugs, nutrition, microbiome, exercise and environmental chemicals.
- Altered programming may alter brain appetite and satiety centers, fat cell numbers, size and function, as well as effects on muscle, GI tract, pancreas and liver.

It was estimated with confidence that:

- Effects of obesogens will be due to “multiple hits” across the lifespan and generations.
- There will be multiple specific windows of enhanced sensitivity across the lifespan including preconception, in utero, neonatal, prepuberty and aging.
- The effects of obesogens will be sexually dimorphic.
- Obesogens must be studied along with nutrition, activity, stress, infections, microbiome etc in order to accurately assess the effects of environent on obesity.
- Some obesogens will act transgenerationally requiring a multigenerational approach.
- The current approach of testing one chemical at a time at one window of exposure is almost certainly underestimating the importance of obesogens.

FUTURE DIRECTIONS

There is an urgent, unmet need to understand the mechanisms underlying the predisposition to obesity and related disorders. Obesity currently adds more than $200 billion to U.S. healthcare costs annually, and the number of obese individuals continues to increase (6). Evidence implicating environmental influences on obesity is mounting, but the mechanisms of environmentally initiated obesity (i.e., other than by foods or lifestyles) remain largely unexplored. For example, while we and others showed that TBT exposure increased adiposity in mice, rats, and zebrafish, our recent study on the effects of altered diet on male F4 descendants of TBT-treated animals is the among the first to test the interactions between diet composition and obesogen exposure (61). Thus, there is an important gap in our understanding of how current or ancestral obesogen exposure interacts with diet, an interaction that could be very significant for human health. Effects of obesogen exposure on MSCs (84; 92) and on mice (61; 85) are likely to be epigenomic, yet little is known about the mechanisms responsible. How are effects of maternal exposure to obesogens transmitted to offspring and to subsequent generations? How does maternal exposure to TBT lead to a transgenerational thrifty phenotype (61) and how are perfluoroalkyl chemicals linked with

reduced resting metabolic rate (62)? Which molecular targets mediate the effects of obesogens on metabolic programming in vivo? A conservative estimate of the impact of three obesogens on the price of obesity in the EU put the annual cost at €18 billion (14); a comparable study estimated the annual cost in the U.S. at $5.9 billion (12). These estimates do not consider the extent to which exposure to organotins, or any of the ~50 other obesogens impacts the human obesity epidemic because there are an insufficient number of prospective cohort studies with multiple measurements of chemical exposure that can be linked with obesity, in vivo. Relatively little is known about the extent to which obesogen exposure programs dysfunctional adipose tissue that may store, but not mobilize fat. There is an extreme paucity of data on the effects of multiple or continuing exposures over the life course and across generations. Almost nothing is known about the extent to which obesogen exposure interacts with other established risk factors in obesity such as inflammation, disrupted circadian rhythms, oxidative stress, mitochondrial dysfunction, dietary composition, timing of eating and the regulation of appetite and satiety. Perhaps most of all, there is an almost complete lack of biomarkers of previous or ancestral obesogen exposure that can be assessed in the numerous existing human cohorts. Identifying these will provide strong insights into the mechanisms underlying the effects of obesogen exposure, who has been exposed and what links can be drawn among these exposures, obesity and metabolic diseases in the population.

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