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MODULATION OF THE MALE REPRODUCTIVE AXIS BY AN ENVIRONMENTAL ANTIANDROGEN DI (2-ETHYLHEXYL) PHTHALATE

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SUMMARY

The endocrine disruptor hypothesis asserts that exposures to environmental agents, which have the ability to perturb the hormonal milieu, exert adverse effects on cellular differentiation and function in affected tissues. These compounds exhibit a predilection for steroid hormone receptors [androgen and estrogen receptors (AR, ER)]. Because ARs and ERs are highly expressed in reproductive tissues, it is not surprising that analysis of reproductive activity following toxicant exposures has received a great deal of attention. We have obtained data describing the effects of an antiandrogen di(2-ethlhexyl) phthalate (DEHP) on the reproductive tract of the male rat. DEHP is the most abundant phthalate in the environment, and phthalates are used to impart flexibility to infant toys, building and food-packaging products, and biomedical devices. DEHP action appears to depend on the time of exposure because prenatal exposures decreased serum luteinizing hormone (LH) and testosterone (T) levels in prepubertal rats while chronic exposures of weanling rats to DEHP caused simultaneous elevations in serum LH and T levels. The pituitary gonadotropin LH is the primary regulator of androgen biosynthesis by Leydig cells in the testis. Taken together, these observations imply that the male reproductive axis is subject to regulation by DEHP. Given the sensitivity of developing organ systems to the action of hormonally active agents, implying increased vulnerability of children to the effects of DEHP, our findings suggest a decrease in the levels of DEHP and other phthalates in consumer products.

INTRODUCTION

There is increasing concern that environmental chemicals may be exerting adverse effects on human endocrine function. Synthetic chemicals have been implicated as potential causes of various human reproductive anomalies (1), including an increase in testicular cancers in young males (2), and increased incidence of cryptorchidism (3) and hypospadias (4). Such development anomalies may associated with excessive estrogenic activity and/or inadequate androgenic stimulation during development, arising from exposure to environmental factors that exert their influence early in life (5).

Ligands, acting as agonists and/or antagonists, that bind the steroid hormone family of nuclear transcription factors, androgen and estrogen receptors (AR, ER), are associated with reproductive anomalies. Androgens are the predominant reproductive hormones in the male, and the primary steroid hormone, testosterone, is produced by Leydig cells in the testis. Prenatally, testosterone stimulates sexual differentiation, and maintains the male phenotype in adulthood.

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Testosterone is also converted to estradiol in the brain resulting in activation of ER-mediated pathways associated with male sexual behaviour (6). There is growing evidence that several chemicals possess the ability to interfere with testosterone-mediated differentiation of the Wolffian duct, dihydrotestosterone-mediated development of the prostate, and/or virilization of the external genitalia during the process of reproductive tract developmet (7, 8). Phthalate esters, widely used in the plastic industry, are credited with antiandrogenicity because they suppress androgen-stimulated sexual differentiation in rodents (9). Di (2-ethylhexyl) phthalate (DEHP), a highly ubiquitous chemical in the environment is widely used in the food-packaging and construction industries and as constituents of biomedical devices (intravenous infusion bags, tubings, catheters etc). The US health care industry alone uses more than 500 million intravenous (iv) bags each year. There is a public health concern that patients undergoing hemodialysis and blood transfusions are exposed to unusually high DEHP levels after it leaches out of PVC medical devices (e.g., iv bags, tubings and blood bags).

Estimates indicate that DEHP is present in indoor air at concentrations ranging from 8 ng/cm^3 to 3 mg/cm^3 (10), and typical human exposure approximates 30 µg/kg/day although occupational and clinical exposures may increase this level to 5 mg (11). Thus, human exposure to DEHP is significant. Following ingestion, lipases in the intestinal epithelium, liver, and other tissues hydrolyze DEHP to its monoester derivative MEHP, which is then widely distributed in the body. MEHP is metabolized further into several other metabolites, including mono- (5-carboxy-2-ethylpentyl) phthalate, mono-(2-ethyl-5-oxohexyl) phthalate and mono-(2-ethyl-5-hydroxyhexyl) phthalate (12, 13).

Steroid hormone receptors (ER and AR) are localized at all levels of the male reproductive axis, including the hypothalamus, pituitary and the testis. Gonadal steroids affect gonadotropin – releasing hormone (GnRH) secretion from the hypothalamus, which regulates pituitary gonadotropin (FSH and LH) secretion by acting on GnRH receptors present in gonadotropes (14). Normally, decreased serum testosterone concentrations, arising from reduced androgen biosynthesis, act via a negative feedback mechanism to induce increased LH output from the pituitary. LH, in turn, stimulates Leydig cells to secrete more testosterone, which in the course of time, and acting via the same pathway, restores pituitary LH release to normal levels. In this manner, the negative feedback mechanism serves as a homeostatic control for the hypothalamo-pituitary-testicular (HPT) axis (15). However, antiandrogenic agents may prevent cellular androgen uptake and/or modulate AR expression and protein levels in target tissues (16). Since the AR, in concert with ERs, mediate negative feedback inhibition of pituitary gonadotropin secretion (17), these effects have the potential to decrease sensitivity of the hypothalamic-pituitary axis to circulating androgen levels, resulting in continuously high LH levels. Pituitary LH is the primary regulator of Leydig cell function, and Leydig cells are the only LH-binding sites in the testis. Thus, modulation of pituitary LH secretion by hormonally active chemicals have implications for Leydig cell development and steroidogenic function.

Early studies of the effects of phthalates on the reproductive axis focused primarily on testicular effects with little information on underlying endocrine changes. There is a large body of data demonstrating that the testicular toxicity of DEHP is mostly associated with the action of its primary metabolite, mono (2-ethylhexyl) phthalate (MEHP), in Sertoli and germ cells (18-20), although disruption of Leydig cell structure and function at a high DEHP dosage level (2g/kg/bw) have beenreported (21). There is evidence that MEHP reduces FSH stimulation of cAMP in Sertoli cells, which may be a contributing factor to DEHP-induced decreases in spermatogenesis and testicular

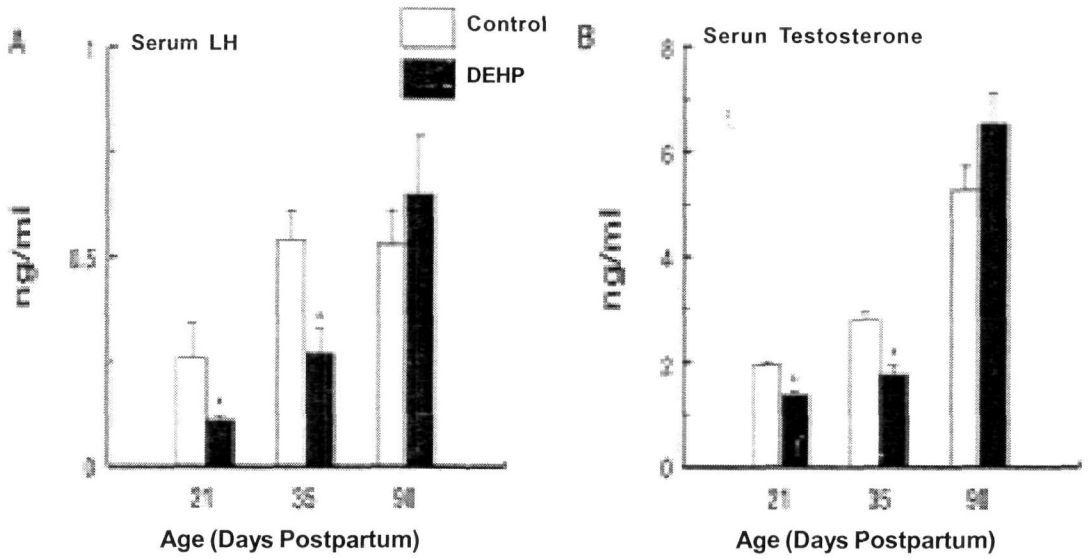

Figure 1. Exposure of pregnant dams to 100 mg/kg/day DEHP from gestation days 12 to 20 decreased the serum concentrations of LH (A) and testosterone (B) in male offspring at 21 and 35 days of age (*p < 0.05). Thease inhibitory effects were, however, no longer apparent at 90 days. *Reprinted with permission from Akingbemi et al., 2001*

size (22, 23). On the other hand, Oishi and Hiraga (24) reported increased testicular testosterone concentrations following DEHP administration. As the rate of spermatogenesis is moderated by the paracrine relationship that exists between Sertoli and Leydig cells (25), it is not known whether changes in androgen biosynthesis contribute to DEHP modulation of Sertoli cell function and spermatogenesis. Unfortunately, complete endocrine evaluations were not undertaken in these studies and it was difficult to discriminate between effects occurring at different levels of the HPT axis. We have conducted experiments *in vivo* to identify DEHP-induced endocrine changes. Specifically, we investigated changes [illegible] cell steroidogenesis.

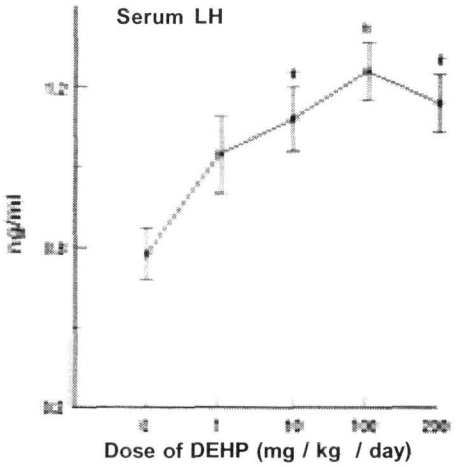

Figure 2a. Chronic DEHP exposure (28 days) were associated with increased serum LH levels (* p < 0.05), with potential implications for Leydig cell mitosis (* p < 0.05). *Reprinted with permission from Akingbemi et al., 2001.*

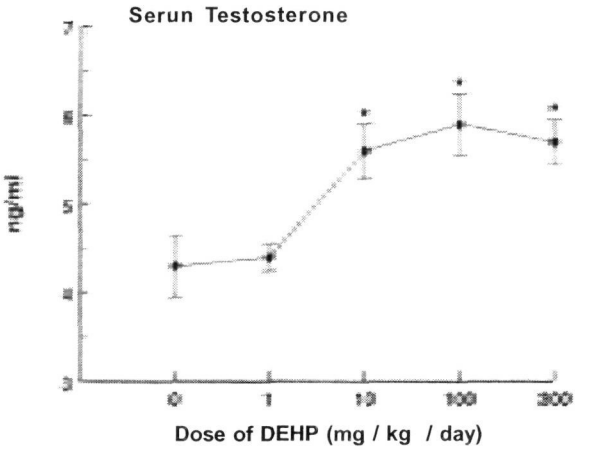

Figure 2b. High serum testosterone levels after DEHP treatment for 28 days were due to enchanced LH stimulation of Leydig cells and increased androgen biosynthesis (* p < 0.05). *Reprinted with permission from Akingbemi et al., 2001.*

Gestational DEHP exposures

Although only small amounts of DEHP ingested by pregnant dams are measured in fetal tissues, DEHP showed a relatively long *in vivo* half-life, implying that repeated maternal exposures during pregnancy might lead to accumulation

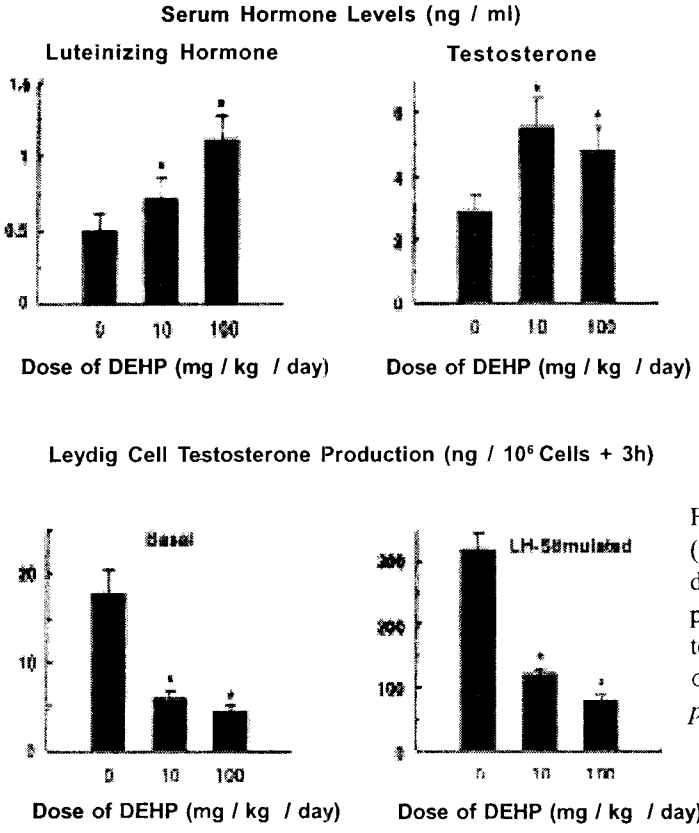

Figure 3. Chronic DEHP exposures of rats (postnatal days 21 to 90, a total of 70 days) decreased Leydig cell androgen production but the serum levels of LH and testosterone were elevated compared to control (* $P < 0.01$). *Reprinted with permission from Akingbemi et al., 2004.*

of toxicologically significant quantities (26). Numerous studies have shown that prenatal exposures to DEHP cause effects in the male offspring in the postnatal period (7, 8, 27). In the present study, male rats exposed to DEHP *in utero* were analyzed at 21, 35 and 90 days of age. At 21 and 35 days, serum LH and T levels were significantly lower in DEHP-exposed rats ($P < 0.05$), compared to control (Fig. 1).

Reduced serum androgen levels were due to a decrease in androgen biosynthesis. However, serum LH and testosterone levels were equivalent in control and DEHP-treated rats when measured at 90 days of age (Fig. 1). These observations show that exposure of male rats to DEHP early in the period of reproductive tract development results in suppression of pituitary LH secretion and a decrease in androgen biosynthesis, however, these effects were no longer apparent in adulthood.

Chronic DEHP exposure in the postnatal period

It is conceivable that exposure of humans to phthalates, including DEHP, occurs over long periods of time. Thus, the effects of chronic exposure to DEHP on Leydig cell steroidogenic function are of interest. In the first set of experiments, prepubertal rats were gavaged with 0, 1, 10, 100 or 200 mg/kg/day DEHP from postnatal day 21 (*i.e.* at weaning) to day 48 (period of 28 days). This exposure

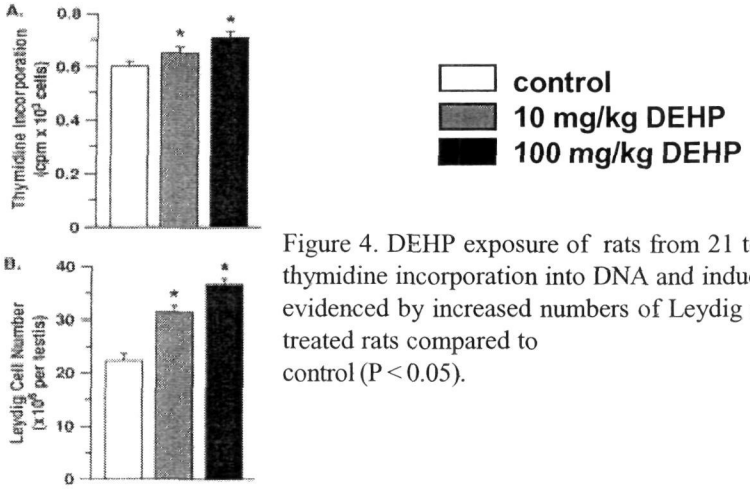

Figure 4. DEHP exposure of rats from 21 to 120 days of age increased thymidine incorporation into DNA and induced Leydig cell hyperplasia, evidenced by increased numbers of Leydig cells in the testis of DEHP-treated rats compared to control ($P < 0.05$).

paradigm enhanced Leydig cell capacity for androgen production in a dose-dependent manner (9). The increase in steroidogenic capacity was associated with higher serum LH and testosterone concentrations (Fig. 2a, 2b). Obviously, the increase in androgen biosynthesis is due to increased LH stimulation of Leydig cells, following an initial decline in testosterone production that was observed after 14-day DEHP exposures (9). However, the simultaneous occurrence of high serum LH and testosterone levels in rats treated with DEHP for 28 days implies a disruption of this mechanism, and suggests that Leydig cells in these animals may be subjected to higher than normal LH stimulation for extended periods of time.

We performed further experiments to test the hypothesis that increased LH stimulation of Leydig cells arising from DEHP-induced elevations in serum LH levels occurring over a long period of time results in premature deterioration of Leydig cell steroidogenic function and cause Leydig cell hyperplasia. Elevated serum LH levels occurring for a prolonged period of time will also have clinical relevance to Leydig cell tumorigenesis as evident in androgen resistant *Tfm* mice that exhibit high serum LH levels and Leydig cell hyperplasia (28). Chronic LH stimulation is known to cause Leydig cell hyperplasia in rodents (29).

Indeed, gonadotropins are considered to be potential tumor promoters because activation of the inositol triphosphate pathway by a mutated LHR gene cause Leydig cell adenomas in humans (30, 31). Therefore, rats were gavaged with DEHP from 21 to 90 days of age and serum LH and testosterone levels were measured at the end of the treatment period. Both parameters were elevated, as occurred after 28 days of treatment, but the steroidogenic capacity of individual Leydig cells was decreased (Fig. 3). The finding of elevated serum LH levels in the presence of higher-than-normal testosterone levels implies that pituitary LH secretion was unresponsive to the increase in peripheral androgen levels. To determine whether sustained increases in serum LH levels stimulated Leydig cell proliferation, thereby increasing Leydig cell numbers, Leydig cells were stereologically enumerated in the testis of DEHP-treated rats *versus* controls. The number of Leydig cells in the testis of DEHP-treated rats was found to be higher than in control animals (Fig. 4). Therfore, the higher serum testosterone levels in the presence of declining steroidogenic capacity is due in part

to an increase in the number of Leydig cells contributing to peripheral androgen levels in DEHP-treated rats.

DEHP effects in humans

A major point of argument has been whether phthalate-induced effects in rodents can be extrapolated to humans. For example, lower toxicity in humans may be related to the following factors : i) phthalate effects are mediated via the peroxisome proliferator-activated receptor α-activated pathway, which is less active in humans (32); ii) primates absorb a smaller percentage of orally administered DEHP and have higher capacities for glucuronidation, and hence excretion, than rodents (46); iii) DEHP has a low affinity for adipose tissue and therefore does not bioaccumulate (47). However, other mechanisms by which DEHP affects testicular function in rats and mice are relevant to humans, e.g., depletion of testicular zinc, alteration of antioxidant status, inhibition of phospholipase A2, and the presence of larger numbers of LH receptors in human Leydig cells (33-35). Children are generally recognized to be at greater risk because chemical-induced alterations *in the hormonal milieu of tissues, cell signaling, growth, histogenesis, and morphogenesis cause* adverse effects in the fetus (36) and hormonally, active chemicals, both xenoestrogens and antiandrogens, exhibit greater potency during sexual differentiation in rodents and humans (37, 38), Due to the smaller body size, it has been suggested that the body burden in children for several environmental chemicals is twice that of adults (39). Thus, given the homology between organ systems in all mammalian species, exposures to DEHP has potential implication for adverse effects in the human, particularly in children.

CONCLUSION

Compounds that alter androgen action act to either inhibit the conversion of testosterone to the more potent androgen DHT (via conversion by the enzyme 5α-reductase), antagonize the binding of androgens to the AR, or suppress androgen biosynthesis (40, 41). Our studies demonstrate that DEHP inhibits androgen biosynthesis by Leydig cells after prenatal exposure or following prolonged exposure in the postnatal period. However, the effects on pituitary LH secretion were in opposite directions, *i.e.*, lower *versus* higher serum LH levels. The presence of reduced LH levels at 21 and 35 days of age after gestational exposure when serum testosterone levels were also suppressed can be interpreted to mean that DEHP suppresses pituitary LH secretion because decreases in serum testosterone levels sought to stimulate increased LH output from the pituitary. On the other hand, we measured concurrent elevations in serum LH and testosterone levels although Leydig cell steroidogenic capacity was diminished following chronic postnatal exposures. These observations could be interpreted to mean that DEHP interferes with the regulatory mechanisms present in the HPT axis. It could also be that DEHP, while suppressing Leydig cell function, had a direct stimulatory effect on pituitary LH secretion. If that were the case, the opposite effects of DEHP on LH secretion in male rats implies that DEHP effects on the pituitary are dependent on the time of exposure. Indeed, hypertrophy of anterior pituitary cells and pituitary tumors occurred after male rats were subjected to high DEHP doses (12 g/kg/day) for a long period of time, 103 weeks (32). Thus, further studies are warranted and are ongoing to test these hypotheses. Another finding of potential interest was the occurrence of higher serum estradiol in DEHP-treated rats *versus* controls during DEHP treatment (42, 46). This agrees with previous observations that chronic LH stimulation also induces aromatase activity in Leydig cells *in vivo* and *in vitro* (43, 44). Since ERs are present in other tissues, e.g., cardiovascular system, bones (45), DEHP-induced increases in estradiol biosynthesis have implications for systemic physiology.

While there has been an increase in the number of reports appearing in the literature on DEHP effects on male reproduction, several issues remain to be addressed. For example, humans are exposed to a variety of chemicals at the same time and possibly for prolonged periods. Thus, it will be useful to develop models that will facilitate evaluation of the effects of chemicals mixtures *in vivo*. Such studies will generate relevant information especially if conducted in models that are more similar to humans than rats and mice, e.g., rabbits and primates. It will also be useful to measure the serum levels of DEHP and its metabolites in rodents and other non-human mammalian species for comparison to phthalate levels measured in human tissues. This approach may further facilitate the process of extrapolation DEHP effects to human for risk assessment.

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