Glucocorticoids Amplify Dibutyl Phthalate-Induced Disruption of Testosterone Production and Male Reproductive Development

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Common male reproductive abnormalities including cryptorchidism, hypospadias, and low sperm counts may comprise a testicular dysgenesis syndrome (TDS), resulting from fetal testis dysfunction during a critical developmental period involving reduced androgen production/action. The recent increase in TDS prevalence suggests environmental/lifestyle factors may be etiologically important. The developing fetus is exposed to multimodal challenges, and we hypothesized that exposure to a combination of factors rather than single agents may be important in the pathogenesis of TDS. We experimentally induced fetal testis dysfunction in rats via treatment of pregnant females daily from embryonic day (e) 13.5 to e21.5 with vehicle, 100 or 500 mg/kg/d dibutyl phthalate (DBP), 0.1 mg/kg · d dexamethasone (Dex), or a combination of DBP + Dex. In adulthood, penile length/normality, testis weight/descent, prostate weight, and plasma testosterone levels were measured plus anogenital distance (AGD) as a measure of androgen action within the masculinization programming window. Intratesticular testosterone and steroidogenic enzyme gene expression were measured in fetal testes at e17.5. High-dose DBP reduced fetal intratesticular testosterone and steroidogenic gene expression; induced mild hypospadias (31%) and cryptorchidism (53%); and reduced penile length, AGD, and testis and prostate weight in adulthood. Dex alone had no effect except to reduce birth weight but amplified the adverse effects of 500 mg/kg · d DBP and exacerbated the effects of 100 mg/kg · d DBP. All adverse effects were highly correlated to AGD, emphasizing the etiological importance of the masculinization programming window. These findings suggest that exposure to common environmental chemicals in combination with, for example, maternal stress, may increase the risk of common male reproductive abnormalities, with implications for human populations. (Endocrinology 150: 5055–5064, 2009)

Abnormalities of male reproductive health including cryptorchidism, hypospadias, testicular germ cell cancer, and subnormal sperm counts are common, and epidemiological evidence suggests that the prevalence of these disorders may be increasing (1). Some studies have reported an increase in incidence of these abnormalities over the past 50 yr, particularly in the West among Caucasians (2–4); however these findings have not been replicated in all reports (5–7), and it is clear that there are striking differences between populations, for example, the incidence of all of these disorders is notably higher in Denmark than Finland (1, 8). The reported rapid changes in prevalence and its geographical restriction, together with evidence from migration studies (9), suggest that environmental and/or lifestyle factors (e.g. exposure to environmental chemicals) may play a key role in the pathogenesis of these

Abbreviations: AGD, Anogenital distance; DBP, dibutyl phthalate; Dex, dexamethasone; e, embryonic day, 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; ITT, intratesticular testosterone; TDS, testicular dysgenesis syndrome.
developmental abnormalities (1, 8), and understanding the factors that may mediate this and their mechanisms of action has major implications for public health.

Cryptorchidism, hypospadias, testicular germ cell cancer, and reduced fertility are all risk factors for each other and may comprise a testicular dysgenesis syndrome (TDS), sharing a common origin in prenatal life (1, 8). It has been proposed that TDS results from a primary abnormality of testis development (dysgenesis) resulting in Leydig and/or Sertoli cell dysfunction with secondary androgen insufficiency and impaired germ cell development during masculinization (1, 8, 10). Studies in rats have demonstrated that androgen action during a critical prenatal period [embryonic day (e)15.5–17.5] is necessary to promote correct development of the male reproductive tract (11) and that cryptorchidism and hypospadias arise from deficient androgen action specifically within this masculinization programming window (11–13). Androgen action during this time may also be a determinant of sperm production/count in adulthood (14). Such a fetal programming window is also likely in humans (between 8 and 14 wk gestation) (11) and nonhuman primates (15, 16). Exposure to environmental or lifestyle factors resulting in disruption of testis development and androgen production during this critical developmental period therefore has the potential to induce abnormal male reproductive development. A number of chemicals that are widespread in the environment (including synthetic chemicals used in solvents and lubricants, plastics, plasticizers, pesticides, fungicides, and pharmaceutical agents) have been proposed to have deleterious effects on male (and female) reproductive development (reviewed in Ref. 17). The effects of many of these substances have been characterized in detail in animal models, and there is some evidence that a number of these compounds may have disruptive effects on reproductive development in humans (17).

Fetal exposure to stress and its glucocorticoid hormone mediators also exerts influences on organ growth, development, and subsequent offspring physiology and pathophysiology (18). In rodents and other mammals including nonhuman primates, prenatal glucocorticoid overexposure as a consequence of maternal stress, treatment with dexamethasone (Dex; a synthetic glucocorticoid that freely crosses the placenta), or inhibition/knockout of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), the placental barrier to physiological glucocorticoids, reduces birth weight and permanently alters offspring physiology (19–21). These consequences of prenatal glucocorticoid overexposure are confined to a specific time window in late gestation (e15.5–21.5), which overlaps with the masculinization programming window (19). Moreover, maternal stress, disease, or malnutrition selectively down-regulates placental 11β-HSD2 (22, 23), suggesting a link between common challenges in the maternal environment and fetal glucocorticoid exposure. These observations have parallels with the effects of stress, glucocorticoids, and malnutrition during human pregnancy on offspring development and subsequent pathophysiology (24–27).

In rats, prenatal glucocorticoid overexposure reduces birth weight (20), itself an established (but unexplained) risk factor for TDS disorders in humans (8, 28), and may also affect reproductive development, including alterations inpubertal timing (29), fetal intratesticular testosterone (ITT), and anogenital distance (AGD; a marker of masculinization) (30, 31).

Many animal studies have used single agents or combinations of similar substances to investigate the mechanisms underpinning male reproductive abnormalities and their potential relationship to environmental exposures; however, in reality a mother and her fetus(es) are exposed to more than a single environmental challenge in pregnancy (17). In rats, for example, exposure of the developing fetus to mixtures of antiandrogenic chemicals at concentrations at which none of the chemicals individually has a significant effect results in major perturbation of androgen-dependent masculinization (32). Additionally, a number of other diverse, but common, environmental agents have been implicated in the increase in male reproductive disorders including maternal exposure to pesticides (33) and cigarette smoke (34). Thus, in reality, the developing fetus is likely to be exposed to multiple different agents or maternal lifestyle risk factors that may impact on reproductive development and multimodal challenges within the expected range may be more important than major changes in a single parameter. In this study we explored how a combination of more clinically relevant fetal exposures might interact to disrupt male reproductive development, using a phthalate ester [dibutyl phthalate (DBP), a compound used in solvents and personal care products], known to induce fetal testis dysfunction and a TDS-like spectrum of disorders in rats, in combination with fetal glucocorticoid overexposure.

**Materials and Methods**

**Animals, treatments, sample collection, processing, and phenotyping**

Wistar rats were maintained in our own animal facility and were fed a soy-free breeding diet [RM3(E) soya free; SDS, Dundee, Scotland]. Housing conditions were carefully controlled [lights on at 0700, off at 1900 h, temperature 19–21 C, GOLD shavings and LITASPIN standard bedding (SPPS, Argenteuil, France)]. Timed females were subjected to the daily treatments described below from e13.5 to e21.5 administered between 0900 and 1030 h. All studies were conducted under licensed approval by the U.K. Home Office guidelines, which also involves an ethical approval step.
Dex (100 mg/kg) a combination of DBP (100 or 500 mg/kg by oral gavage) plus

Endocrinology, November 2009, 150(11):5055–5064 endo.endojournals.org

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gene expression in fetal life and plasma

Determination of ITT and steroidogenic enzyme
programming window. Fetuses were removed, decapitated, and
were killed by inhalation of carbon dioxide on e17.5, having
offspring were allowed to grow to adulthood (no. of litters) Birth weight (g)

<table>
<thead>
<tr>
<th>In utero treatment</th>
<th>No of animals of litters</th>
<th>Birth weight (g)</th>
<th>Cryptorchid animals, %</th>
<th>Animals with hypospadias, %</th>
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<tr>
<td>Control (vehicle)</td>
<td>40 (14)</td>
<td>5.84 ± 0.07 (100%)</td>
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<td>Dex-100 µg/kg</td>
<td>35 (12)</td>
<td>5.07 ± 0.08 (87%)</td>
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<td>DBP-100 mg/kg</td>
<td>45 (15)</td>
<td>5.80 ± 0.08 (99%)</td>
<td>0</td>
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<tr>
<td>DBP-500 mg/kg</td>
<td>32 (13)</td>
<td>5.40 ± 0.16 (92%)</td>
<td>53b</td>
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<td>Dex-100 µg/kg + DBP-100 mg/kg</td>
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χ² testing revealed an effect of Dex+DBP-500 mg/kg exposure to significantly increase the severity of hypospadias (P = 0.003).
a Means ± SEM (values in parentheses show mean as percent of control value).
b P < 0.0001, in comparison with control (χ² test).
c P < 0.0001, in comparison with respective DBP-only group.
d P = 0.004, in comparison with respective DBP-500 mg/kg-only group (χ² test).

Treatments included the following: 1) DBP (Sigma-Aldrich Co. Ltd., Dorset, UK) at a dose of either 100 or 500 mg/kg administered by oral gavage in 1 ml/kg corn oil, plus daily subcutaneous injection of vehicle; 2) Dex (Sigma-Aldrich Co. Ltd., Dorset, UK) at a dose of either 100 or 500 mg/kg administered by oral gavage in 1 ml/kg corn oil, plus daily subcutaneous injection of vehicle; 3) a combination of DBP (100 or 500 mg/kg by oral gavage) plus Dex (100 µg/kg · d sc); 4) control (vehicle by gavage and sc injection); and 5) DBP (500 mg/kg) + Dex (100 µg/kg) from e13.5 to e17.5 to confine treatment to the masculinization programming window.

The number of pregnant animals treated and the numbers of male offspring evaluated in adulthood are listed in Table 1. The doses of DBP and Dex were based on our previous studies. Male offspring were allowed to grow to adulthood (>12 wk of age), when they were killed by inhalation of carbon dioxide and blood collected by cardiac puncture into a heparinized syringe for subsequent determination of plasma testosterone levels. Animals were subjected to a thorough inspection to determine the normality of the penis and testicular descent. Hypospadias was classified as mild, moderate, or severe (11); severe cases were all perineal and included complete exposure of the penile os bone. Cryptorchidism was first determined after opening of the abdomen; testes were classified as inguinal (at around the level of the bladder) or high abdominal (adjacent to the kidney). AGD was measured before opening of the abdomen, using digital calipers (Faithfull Tools, Kent, UK) as an additional parameter to AGD in individuals to be assessed. However, anal-
placed in ice-cold PBS (Sigma-Aldrich). Testes were removed via microdissection, snap frozen, and stored at −70 C for subsequent gene expression or testosterone analysis. For ITT, pools of two testes were homogenized and extracted to ensure that levels were detectable. Fetal ITT and plasma testosterone levels in adults were measured after extraction twice with 10 vol hexane-ether [4:1 (vol:vol)] and the organic phase dried down under N₂ at 55 C. The intra- and interassay coefficients of variation were less than 9% and less than 14%, respectively, for plasma, and the intraassay coefficient of variation for ITT was less than 13% (all samples were run in one assay); the limit of detection of the testosterone assay was 40 pg. Other details were as described previously (35).

For quantitative analysis of gene expression by RT-PCR, total RNA was extracted from single e17.5 testes from four of the treatment groups (controls, Dex, DBP-500 mg/kg, Dex + DBP-500 mg/kg) using the RNaseasy micro kit with on-column DNase digestion (QIAGEN, Crawley, UK). Random hexamer primed cDNA was prepared using the Applied Biosystems Taqman reverse transcriptase kit (Applied Biosystems, Warrington, UK). Quantitative real time PCR was performed on the ABI Prism sequence detection system (Applied Biosystems). Expression of rat STAR and Cyp11a1 mRNA was determined using the Roche Universal Probe Library (Roche Diagnostics, Ltd., Burgess Hill, UK) (STAR forward primer: 5’-TCACGTGGCTGCTCAGTATT-3’, reverse primer: 5’-GGGGTCGTGTGAAGACTTGTTG-3’, probe no. 83, catalog no. 04689062001; Cyp11a1 forward primer: 5’-TAT-TTCCGGTTGCGGTTAG-3’, reverse primer 5’-CAGCATCTCCTCAAGATCC-3’, probe no. 9, catalog no. 04685075001; Roche Applied Sciences, Burgess Hill, UK). The expression levels of both STAR and Cyp11a1 were corrected using a ribosomal 18S internal control (Applied Biosystems catalog no. 4308329). All samples were performed in triplicate and a relative comparison was made to adult testis control cDNA. Analyses were undertaken for at least 12 e17.5 rat fetuses from at least four litters per group.

**Table 1. Animal treatments and numbers, birth weight, and incidence in adulthood of cryptorchidism and hypospadias**

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**Determination of ITT and steroidogenic enzyme gene expression in fetal life and plasma testosterone in adulthood**

In a further study, pregnant dams from treatment groups 1–4 were killed by inhalation of carbon dioxide on e17.5, having received their last treatment around 24 h earlier. This time point was chosen because it is at the end of the masculinization programming window. Fetuses were removed, decapitated, and
ysis of litter means for treatment effects yielded similar results, although usually with lower significance levels. Data for ITT at e17.5, which will determine AGD, is presented and analyzed for both individuals and litter means to illustrate this. All statistical analyses used GraphPad Prism (version 5; GraphPad Software Inc., San Diego, CA). Data were analyzed using one-way ANOVA followed by the Bonferroni post hoc test, comparing all single and combined treatments with controls and with each other. The χ² test was used for comparing incidence and/or severity of cryptorchidism and hypospadias among treatment groups. Data for fetal testicular gene expression and testosterone content and adult plasma testosterone levels were log transformed before analysis to normalize variances.

Results

Effects on birth weight and general observations
Male offspring born to mothers treated with DBP-500 mg/kg had reduced birth weight (8% reduction) (Table 1), whereas those exposed to DBP-100 mg/kg had birth weights comparable to controls. Males born to mothers treated with Dex had a more pronounced reduction (13%) in birth weight. Combined treatment with Dex plus either DBP-100 mg/kg or DBP-500 mg/kg resulted in notably greater reductions in birth weight (21 and 28%, respectively), effects that were highly significant when compared with the respective single treatments (Table 1). Despite these reductions in birth weight, there was no obvious general ill health, and by 6 months of age, there were no differences in body weight between any of the treatment groups (data not shown). Treatment effects on birth weight did not show any relationship to alterations in reproductive development as described below.

Effect on AGD in adulthood
Gestational exposure to DBP-500 mg/kg, but to neither DBP-100 mg/kg nor Dex alone, reduced AGD (Fig. 1). Combined treatment with Dex plus either DBP dose significantly reduced AGD compared with controls, and for the higher DBP dose, this reduction was greater (P < 0.001) than after treatment with DBP-500 mg/kg alone (Fig. 1). Confining treatment with DBP-500 mg/kg + Dex to the male programming window (e13.5–17.5) resulted in a similar effect on AGD in adulthood (31.3 ± 0.7 mm; mean ± SEM, n = 7) as treatment from e13.5–21.5, confirming that AGD can be used as a measure of fetal androgen exposure specifically during the masculinization programming window.

Effect on penis length, prostate and testis weights, and plasma testosterone levels in adulthood and relationship to AGD
Whereas fetal exposure to DBP-100 mg/kg or Dex alone had no effects on testis weight, ventral prostate weight, or penis length, DBP-500 mg/kg significantly reduced all three parameters (Fig. 2). Additional exposure to Dex exacerbated the DBP-500 mg/kg-mediated reduction in adult testicular weight and resulted in reduced testicular weight with DBP-100 mg/kg compared with controls. These effects were mirrored by reductions in plasma testosterone in DBP + Dex groups compared with DBP treatment alone. Dex exposure did not significantly alter DBP-500 mg/kg effects on penis length or ventral prostate weight.

To establish whether the treatment-induced changes in the parameters shown in Fig. 2 were potentially related to deficient androgen action within the masculinization programming window, the relationship with AGD was evaluated using linear regression (Fig. 3). This indicated that all four parameters were significantly correlated with AGD. This association was modest for plasma testosterone levels but was pronounced for penis length and especially for prostate and testis weights (Fig. 3).

Effects on incidence and severity of hypospadias and cryptorchidism and relationship to AGD
Cryptorchidism or hypospadias were not observed in offspring from control or DBP-100 mg/kg- or Dex-exposed pregnancies (Table 1). However, maternal exposure to DBP-500 mg/kg was associated with both malformations. Importantly, the addition of Dex increased the incidence of cryptorchidism observed after DBP-500 mg/kg and also significantly increased the severity of hypospadias from mainly mild to predominantly severe (Table 1).

The occurrence of any severity of hypospadias or cryptorchidism was associated with significantly lower AGD in affected animals when compared with animals with scrotal testes (the latter group consisted of animals derived from all treatment groups). Although there was a stepwise reduction in AGD with increasing severity of hypo-
spadias, there were no statistically significant differences in AGD among the three hypospadias categories (Fig. 4). In contrast, the occurrence of both cryptorchid testes in a high abdominal position, viewed as being the most complete inhibition of testicular descent, was associated with a significantly lower AGD \((P < 0.01)\) than for any of the other three cryptorchidism categories (Fig. 4).

**Effects on androgen production (ITT) and steroidogenic gene expression in fetal life**

Because the results above suggested that treatment effects on reproductive organs were potentially related to decreased androgen action in the masculinization programming window (based on AGD), we sought to confirm this directly by measuring ITT content and mRNA expression of two key testicular steroidogenic genes \((\text{StAR} \text{ and Cyp}11a1)\) at e17.5 (at the end of the masculinization programming window). Treatment with DBP-500 mg/kg reduced fetal testicular ITT, \text{StAR}, \text{ and Cyp}11a1 mRNA expression (Fig. 5). Whereas Dex alone had no effect on these parameters, combined treatment with Dex + DBP-500 mg/kg resulted in more pronounced suppression of ITT and \text{StAR} and \text{Cyp}11a1 mRNA expression than DBP-500 mg/kg alone (Fig. 5).

**Discussion**

The present study is the first to have examined the effect of an agent (DBP) that disrupts fetal testicular testosterone production in combination with a second hit, prenatal glucocorticoid overexposure. Fetal glucocorticoid overexposure increased the severity of DBP-induced effects on AGD, testis weight, and plasma testosterone in adulthood and markedly increased the rate of cryptorchidism and the severity of hypospadias. Additionally, glucocorticoid overexposure revealed effects on AGD and testis weight at a lower dose (100 mg/kg) of DBP.

The role of environmental chemicals in the pathogenesis of reproductive disorders remains controversial. Evidence from human populations is partial and indirect (17), and existing human studies have tended to focus on exposure to a single compound or a family of substances rather than on the complex mixtures of substances that have the potential to disrupt reproductive development to which humans are undoubtedly exposed (17). Many epidemiological studies have demonstrated that events in early life (pre- or early postnatal) can have a profound influence on disease risk in later life (36), but it is clear that the time course between exposure and the manifestation of the effects of such an exposure can be many years (8, 37), so that observational stud-
ies linking early life events or exposures with later disease risk in humans are difficult.

Animal studies demonstrating that environmental chemicals (including phthalates) can disrupt reproductive development have therefore been useful to delineate the mechanisms by which early life exposures cause later disease; however, these studies required relatively large doses to produce the phenotype. Thus, in terms of phthalate exposure, although there is widespread human exposure to a range of phthalates with generally higher exposures in infants than adults and in women than men (38, 39), it is unclear whether the human fetus is exposed to sufficient levels of such chemicals to result in any adverse effect.

Studies of the relationship between phthalate exposure and male reproductive parameters in humans are not conclusive; in some human studies in the United States, maternal exposure to phthalates in pregnancy has been found to be associated with reduced AGD and penis size and with cryptorchidism (40, 41). In contrast, another study in Denmark and Finland, which measured phthalate levels in breast milk found no association with cryptorchidism but did report levels of a phthalate metabolite (monobutyl phthalate) to be associated with lower free testosterone levels in male infants aged 1–3 months (42). Finally, another small study in Taiwan failed to find any relationship between phthalate exposure and AGD in boys (43). Nevertheless, if the associations between phthalate exposure and disrupted reproductive development are indeed true, and if they are causal, they indicate that urinary phthalate levels in pregnant women in association with reduced AGD in their male offspring are within the range reported in the general population (44), exposure levels that are considerably lower than those required to induce TDS disorders in rats.

Part of the explanation for this might lie in the fact that levels of phthalate metabolites in amniotic fluid in human pregnancies are around 20% of those in amniotic fluid of rat fetuses of mothers exposed to DBP-100 mg/kg (45, 46), and given that the masculinization programming window in which exposure is critical to induce a TDS phenotype is only 3–4 d in rats, whereas it may be as long as 4–6 wk in humans (11), the cumulative exposure in human fetuses may well be equivalent or even greater than that seen in rodents. Additionally, humans are exposed to a number of different phthalates that can induce TDS-like disorders in rats, and it is well established that additive effects of these compounds occur when administered together to pregnant rats (47, 48). Thus, the doses used in rodent experiments may have relevance to human exposures.

Although the precise role of phthalates in disrupting reproductive development in humans remains unclear, we chose to use DBP in this study as an exemplar agent because of its well-described effects in rats to induce fetal testis dysfunction and a TDS-like spectrum of disorders in association with a marked reduction in fetal Leydig cell hormone (testosterone and insulin-like factor 3) production (35, 49–52). Thus, the adult consequences of fetal DBP exposure reported here are in agreement with previous studies (14, 35, 49, 50, 53). Our findings therefore provide strong support for the concept that TDS disorders have their origins within a critical prenatal developmental period (11) and refine it by showing that altered androgen action within the masculinization programming window in rats appears to be of paramount importance in determining abnormalities of penis development (reduced length, hypospadias), testis descent and adult testis size (and thus sperm production/count), and prostate size. Additionally, DBP exposure through to e21.5 had no greater suppressive effect on AGD than when exposure was confined to e13.5–17.5, demonstrating that AGD can be used as a simple, noninvasive guide to the level of androgen action specifically within the masculinization programming window (11). Our results also imply that AGD measurement at birth could provide a means of predicting the reproductive phenotype (reproductive organ size, occurrence of abnormalities) of an individual in adulthood.
because other studies in rats have shown that AGD at birth is highly predictive of abnormalities after exposure in fetal life to the antiandrogen, flutamide (54).

Crucially, glucocorticoid overexposure, whereas having no effect on reproductive development when used alone, amplified the adverse effects of an agent disrupting fetal testicular development. Furthermore, the degree of suppression of fetal ITT matched the severity of reproductive disorders/deficits observed in adulthood, including reduced testis, prostate, and penis size, the occurrence of cryptorchidism and the severity of hypospadias. This implies that stress or malnutrition (which attenuates the placental 11β-HSD2 barrier to maternal glucocorticoids) in pregnancy may reveal TDS spectrum disorders in human populations exposed to numerous chemicals with antiandrogenic activity (32, 33). How glucocorticoids amplify antiandrogenic effects in the fetal testis/reproductive tract remains to be determined, but fetal glucocorticoid exposure alters proliferation and function of the adult Leydig cell population (55, 56), perhaps by down-regulating steroidogenic enzyme expression (57), key nodes in the effects of androgens on testicular development (58). Although recent reviews have suggested that there is an urgent need to consider the toxicological effects of pharmaceutical agents and endocrine-disrupting chemicals on the hypothalamic-pituitary-adrenal axis (17, 59), this is the first study to report that interactions between glucocorticoids and environmental chemicals known to have an effect on reproductive development may play a causative role in increasing disease susceptibility.

Low birth weight is known to be a risk factor for TDS disorders in humans, although the reasons for this are unclear (8, 28). We were unable to show that restricted fetal growth per se, resulting from fetal glucocorticoid overexposure, affects AGD or causes TDS disorders in rats and exposure to DBP was associated with a mild reduction in birth weight only at the higher dose. However, exposure to a combination of Dex and DBP (at either dose) reduced birth weight to a much greater extent, but this was not associated directly with the occurrence of abnormalities. Nevertheless, if our findings in rats are directly relevant to humans, they imply that the increased risk of TDS disorders resulting from restricted fetal growth (28) could potentially alter susceptibility to fetal testis disruption by other lifestyle or environmental factors.

In human studies, low birth weight is associated with an increased risk of cardiovascular disease in later life (60), and in rats, prenatal glucocorticoid overexposure reduces birth weight and is associated with insulin resistance, glucose intolerance, and hypertension in adulthood (19, 61) so that exposure to multiple agents in utero could also potentially be associated with increased cardiovascular risk in adulthood. However, little is known about the metabolic sequelae of prenatal phthalate exposure in animals or humans (61, 62), although there are clear associations in adult men between testosterone levels, adiposity, and cardiometabolic disease (63, 64). This raises the possibility that early life exposure to environmental chemicals affecting the levels of sex steroids and reproductive development could also affect predisposition to obesity and cardiometabolic disease in later life.

**FIG. 5.** Effects on androgen production and steroidogenic gene expression in fetal life (e17.5) in individual male fetuses and litter means. A and B, ITT levels after in utero exposure to vehicle (control), DBP (500 mg/kg • d), Dex (100 μg/kg • d), or DBP + Dex. C and D, mRNA expression of cholesterol side-chain cleavage enzyme (Cyp11a1). E and F, mRNA expression of steroidogenic acute regulatory protein (StAR). In each case, treatment was from e13.5 to e16.5. The data are illustrated as means ± SEM for individual fetuses (n = 8–17 animals/group) or as litter means (one to five animals per four to five litters per group). *, P < 0.05, **, P < 0.01, ***, P < 0.001, in comparison with respective control value. Other comparisons are indicated by the capped lines. NS, Not significant.
One unexplained observation from the present and from our previous (unpublished) studies is that fetal exposure to DBP-100 mg/kg resulted in significantly elevated plasma testosterone levels in adulthood. Whether this involves altered Leydig cell numbers, function, or altered hypothalamic-pituitary activity is under investigation. Other studies (65) also suggest that similar doses of another phthalate may have positive effects on postnatal Leydig cell function, and a recent study has shown that even in fetal life (e21.5), exposure to DBP 100 mg/kg may cause a rebound increase (at 48 h) in ITT levels after initially causing suppression (66). These observations suggest that phthalate effects on Leydig cell function in rats may not always be negative.

In conclusion, our observations in a new model of key biological relevance provide strong support for the TDS concept and show that combinations of exposures during a critical developmental period may be of crucial importance in determining the risk of TDS disorders. We show that these exposures may not be limited to substances commonly proposed as potential mediators of reproductive dysfunction. We propose that adverse effects on male reproductive development and fertility may result from altered susceptibility to fetal testis disruption by combinations of lifestyle and environmental factors and suggest that there is an urgent need to explore the interactive effects of combinations of diverse exposures on disease risk in both animal models and human studies.

Acknowledgments

We thank Mark Fisken and William Mungall for technical assistance.

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This work was supported by Grant FP7-ENV-2007-1-212844 from the European Union (FP7-ENV-2007-1-212844) and Grant U.1276.00.003.0003.01 from the U.K. Medical Research Council and G0501904 (to A.J.D.).

Disclosure Summary: The authors have nothing to declare.

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