In utero Exposure to Low Levels of the Ubiquitous Plasticizer Di-(2-Ethylhexyl) Phthalate Predisposes the Adult Male Sprague-Dawley Adrenal Gland for Endocrine Disruption

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Abstract

Di-(2-ethylhexyl) phthalate (DEHP) is a plasticizer commonly used in the production of polyvinyl chloride products. Because DEHP is not covalently bound to the polymer, it is able to leach out and has been identified as a ubiquitous contaminant in the environment. In humans, DEHP and its metabolites have been detected in umbilical cord blood and amniotic fluid suggesting exposure begins early in fetal development. To study the *in utero* effects of DEHP, we used an animal model where pregnant dams were gavaged with DEHP from gestational day (GD) 14 to birth. GD14 is used in particular as it marks a key time point in the development of the male rat where the adrenogonadal primordium has differentiated but the sexual tissues have not matured. We previously identified that exposure to >100 mg DEHP/kg body weight (BW)/day reduced serum levels of testosterone and aldosterone in the adult male offspring. Furthermore, transcriptomic and epigenomic work in the adrenal glands identified changes in gene expression and DNA methylation and doses as low as 1 mg DEHP/kg BW/day. This suggested that even an environmentally-relevant dose of DEHP may affect the adrenal glands, making it vulnerable to adverse from a secondary stressor later in life. In the present study, we exposed males *in utero* to 1 mg DEHP/kg BW/day and examined global gene expression in the zona glomerulosa. Interestingly, many of the deregulated genes we found were associated with pathways that were deregulated at the 100 and 300 mg doses including immune pathways, lipid

and lipoprotein metabolism, peroxisome proliferator-activated receptor (PPAR) signaling, and mitogen-activated protein kinase signaling pathways. We further treated these DEHP-exposed males with various stressor compounds from post-natal day 54 – 59. Specific treatment with the PPARγ antagonist, T0070907, resulted in a significant decrease of serum aldosterone in males exposed to the 1 mg dose. Additionally, gene expression studies of the adrenal revealed altered expression of the potassium channel, *Kcnk5*, as well as the two nuclear receptors, *Rxrα* and *Rxrβ*. Together, these results suggest *in utero* exposure to environmentally-relevant doses of DEHP predisposes the adult zona glomerulosa to endocrine disruption.

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Table of Contents

List of Abbreviations

ACE	Angiotensin-converting enzyme
AGD	Anogenital distance
AGP	Adrenogonadal primordium
ATII	Angiotensin II
AGTR1	Angiotensin II receptor type 1
AGTR2	Angiotensin II receptor type 2
BW	Body weight
CYP11A1	Cytochrome P450 Family 11 Subfamily A Member 1
CYP11B2	Cytochrome P450 Family 11 Subfamily B Member 2
DAX1	Dosage-sensitive sex reversal gene 1
DEHP	Di-(2-ethylhexyl) phthalate
EDC	Endocrine-disrupting chemical
FABP4	Fatty acid binding protein 4
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GD	Gestational day
HMGCR	HMG-CoA reductase
HMGCS1	HMG-CoA synthase
HSL	Hormone-sensitive lipase
INSIG1	Insulin-induced gene 1
KCNK5	Potassium two pore domain channel subfamily K member 5
LDLR	Low density lipoprotein receptor
LIPE	Lipase E, hormone sensitive

MAPK	Mitogen-activated protein kinase
MEHP	Mono-(2-ethylhexyl) phthalate
MGLL	Monoglyceride lipase
MR	Mineralocorticoid receptor
NR4A1	Nuclear receptor subfamily 4 group A member 1
NR4A3	Nuclear receptor subfamily 4 group A member 3
PLIN	Perilipin 1
PND	Post-natal day
PPARα	Peroxisome proliferator-activated receptor alpha
PPARδ	Peroxisome proliferator-activated receptor delta
PPARγ	Peroxisome proliferator-activated receptor gamma
PRKACB	Protein kinase cAMP-activated catalytic subunit beta
QPCR	Quantitative polymerase chain reaction
RAAS	Renin-angiotensin-aldosterone system
RXRα	Retinoid-X receptor alpha
RXRβ	Retinoid-X receptor beta
RXRγ	Retinoid-X receptor gamma
SCARB1	Scavenger receptor class B member 1
SF1	Steroidogenic factor 1
STAR	Steroidogenic acute regulatory protein
TDS	Testicular dysgenesis syndrome
TSPO	Translocator protein

List of Figures

List of Tables

Introduction

The Adrenal Gland

Adrenal development

The adrenal glands and gonads originate from a common fetal structure – the adrenogonadal primordium (AGP). In rats, this structure is first detected at gestational day (GD) 11.5, marked by the expression of steroidogenic factor 1 (SF1/NR5A1) [1]. The AGP begins to separate at GD12.5 and results in distinct adrenal and gonadal cell populations by GD13.5. From GD13 – 15, neural crest cells migrate to the fetal adrenal cortex where they exist as islands of chromaffin cells and will ultimately give rise to the chromaffin cells of the adrenal medulla [2]. By GD16, the fetal adrenal separates from the surrounding mesenchyme and completes the encapsulation process. Soon after, the definitive zone emerges between the capsule and the growing fetal zone of the cortex. Shortly after birth, the adrenal undergoes zonation resulting in the aggregation of chromaffin islands in the medulla and the regression of the fetal zone. From post-natal day (PND) 10 – 20 is the rise of the rodent-specific X-zone. In males, the X-zone persists until puberty whereas in females, it regresses during the first pregnancy [3, 4]. Postnatal development of the definitive zone will eventually give rise to the zona glomerulosa (ZG), zona fasciculata (ZF), and the zona reticularis (ZR) [5, 6].

The proper development of the adrenal is driven by a number of key genes. The *Wt1* tumor suppressor is one such gene that has been identified to play a crucial role in the proper development of the urogenital ridge which gives rise to the adrenogonadal primordium and the kidneys. Homozygous mutation of this gene in mice is embryonic lethal and results in failure of kidney, gonadal, and adrenal development [7]. Other genes such as *Sf1* and *Dax1* (*Nr0b1*) are involved further downstream in the development of the adrenogonadal primordium. *Dax1*

knockout studies in mice reveal retention of the X-zone as well as complete loss of the germ cell population in males [8]. In humans, mutation or deletion of *Dax1* is thought to be responsible for X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism [9]. Knockout of *Sf1* in mice results in adrenal and gonadal agenesis similar to the phenotype seen in loss of *Wt1* [10]. Mutations in *Sf1* in humans cause similar problems including adrenal failure and sex reversal in males [11]. Other genes including *Ngfib* (*Nr4a1*) and *Gata6* are thought to be important in proper adrenal zonation and expression of steroidogenic enzymes [12].

Steroidogenesis

Cholesterol is the basis for the biosynthesis of all steroids (Figure 1). In the rat adrenal, up to 80% of cholesterol used in steroidogenesis is obtained from extracellular sources [13, 14]. The majority of this circulating cholesterol is found in high-density lipoproteins which is imported into the cell through the scavenger receptor class B member 1 (SCARB1) pathway [15]. Additional circulating cholesterol can be found in low-density lipoproteins which is imported via low-density lipoprotein receptor (LDLR)-mediated endocytosis [16]. Lipoproteins from the endocytic pathway undergo hydrolysis by lysosomal lipases resulting in free cholesterol ready for steroid biosynthesis while cholesterol from the SCARB1 pathway must first be de-esterified by the hormone-sensitive lipase (HSL) before entering the pool of free cholesterol. The remaining 20% of cholesterol used for steroidogenesis is made available through *de novo* synthesis [17] or from excess cholesterol previously stored in intracellular lipid droplets in the form of cholesteryl esters and utilized through the action of HSL [15, 18].

The first phase of steroidogenesis involves the transport of intracellular cholesterol into the mitochondria and is known to be the rate-limiting step in steroid production. Cholesterol is

initially targeted to the outer-mitochondrial membrane by the steroidogenic acute regulatory protein (STAR). Here, it is transported to the inner-mitochondrial membrane through the action of a protein complex which includes the translocator protein (TSPO), the voltage-dependent anion channel (VDAC), and the AAA+ ATPase, ATAD3 where it is converted to pregnenolone by the cytochrome P450 side chain cleavage enzyme (CYP11A1) [19-21]. Pregnenolone, the precursor to all steroids, is free to diffuse out to other steroidogenic organelles for further modifications. In the second phase of steroidogenesis, the final steroid product is determined by cell-specific expression of steroidogenic enzymes. In the adrenal gland, the ZG and ZF are responsible for the production of mineralocorticoids and glucocorticoids respectively. Adrenal expression of enzymes including CYP11B1 and CYP11B2 results in the conversion of precursors into cortisol and aldosterone [22]. Lack of CYP17A1 expression in rodents leads to corticosterone being the major glucocorticoid [23, 24] as well as the lack of dehydroepiandrosterone and dehydroepiandrosterone sulfate production in the ZR of the adrenals [25].

Other major organs of steroid production within the body include the testis and ovary. Leydig cells within the testis are the site of production of the male sex hormone, testosterone. Similarly, theca and granulosa cells within the ovary, as well as the placenta during pregnancy, are responsible for the production of the female sex hormones, estrogen and progesterone.

Steroid hormone receptors

The action of steroid hormones is mediated primarily through intracellular steroid nuclear receptors. Steroid hormone receptors are grouped in subfamily 3 of the superfamily of nuclear receptors. Subfamily 3A includes the estrogen receptors α (ERα; NR3A1) and β (ERβ; NR3A2)

while subfamily 3C contains the glucocorticoid receptor (GR; NR3C1), mineralocorticoid receptor (MR; NR3C2), progesterone receptor (PR; NR3C3), and androgen receptor (AR; NR3C4). MR, like other steroid hormone receptors, is localized to the cytoplasm in the absence of a steroid ligand. Upon mineralocorticoid binding, MR homodimerizes and translocates to the nucleus where it binds hormone response elements within the DNA to regulate transcription [26]. Interestingly, in addition to aldosterone and deoxycorticosterone (IC_{50} = 1.5nM and 3.9nM respectively), MR binds with high affinity to glucocorticoids (cortisol IC_{50} = 2.1nM; corticosterone IC_{50} = 1.2nM) and progesterone (IC_{50} = 1.0nM) [27]. Specificity of MR to aldosterone is maintained by tissue expression of 11β-HSD2 and other enzymes which converts active cortisol to inactive cortisone and progesterone to weaker affinity metabolites [28]. In addition, it has been shown that aldosterone dissociates more slowly from MR when compared to cortisol suggesting the MR is able to distinguish between the bound ligands [29, 30].

The mineralocorticoid action of aldosterone results in an increase of sodium reabsorption by affecting epithelial Na^+ channels (ENaC) and Na^+/K^+ ATPases in a number of tissues including the kidney and colon [31]. This process of increased sodium reabsorption is thought to occur in two phases. The early phase is thought to be driven by expression of the serum and glucocorticoid-regulated kinase which increases the sodium transport activity of existing ENaCs [32, 33]. The late phase is thought to involve increased translation of the various ENaC subunits in order to increase sodium transport [34, 35].

Renin-angiotensin-aldosterone system

The zona glomerulosa-secreted hormone aldosterone acts on the principal cells of the distal convoluted tubule and collecting ducts to increase sodium reabsorption and potassium excretion

[36, 37]. This effect ultimately results in changes to blood volume, blood pressure, and serum electrolyte concentrations. The production of aldosterone by the adrenal gland is primarily regulated by the renin-angiotensin-aldosterone system (RAAS) (Figure 2). When the cells of the macula densa detect a reduction of renal perfusion, it signals the juxtaglomerular cells to release the enzyme, renin. Circulating renin then acts on the liver-made protein, angiotensinogen to produce the 10 a.a. peptide, angiotensin I. Angiotensin I is further cleaved in the lungs by the angiotensin converting enzyme (ACE) to produce angiotensin II (ATII) which binds angiotensin II receptors (AGTR) throughout the body. The AGTR is divided into two subtypes, AGTR1 and AGTR2. The aldosterone stimulation and vasoconstriction effects of ATII are both mediated through AGTR1 found in the adrenal cortex and smooth muscle respectively. Although the role of AGTR2 is not fully understood, its expression has been detected in the adrenal medulla and the brain [38, 39]. In rodents, the AGTR1 is further divided into AGTR1a and AGTR1b [40]. While AGTR1a is found in many tissues throughout the body, AGTR1b is detected only in the brain, testes, and adrenal glands. A double knockout of both subtypes in mice results in aberrant kidney morphology and hypotension, a phenotype similar to homozygous mice with a mutation in the angiotensinogen gene [41, 42].

In addition to the RAAS, aldosterone production is stimulated by increases in serum potassium. Of the potassium channels expressed in the zona glomerulosa, the TWIK-related acid-sensitive potassium channels, TASK1 (KCNK3) and TASK3 (KCNK9), play an important role in maintaining the resting membrane potential and are thought to detect changes in potassium levels [43, 44]. TASK1 knockout studies in mice reveal hyperaldosteronism caused by defects in adrenal cortex zonation further suggesting a role in adrenal development and aldosterone regulation [45].

Di-(2-ethylhexyl) Phthalate

Phthalates

Di-(2-ethylhexyl) phthalate (DEHP) is a plasticizer used in the production of polyvinyl chloride plastics to increase overall strength and malleability of the final product. DEHP is the most commonly used phthalate with up to 3 million tons produced annually [46]. DEHP is detected ubiquitously in the environment and forms part of furniture, flooring, cosmetics, clothing, toys, detergents, automobiles, plastic films, and medical equipment including medical tubing and blood bags [47-50]. DEHP can comprise up to 40% of the total product by weight.

Phthalates and their metabolites have been shown to exert their action through the peroxisome proliferator-activated receptor (PPAR) pathway [51]. PPARs heterodimerize with the retinoid X receptors (RXRs) and bind to peroxisome proliferator response elements (PPREs) in the DNA to regulate downstream gene expression. Mono-(2-ethylhexyl) phthalate (MEHP), the bioactive metabolite of DEHP, in particular, has been shown to bind and activate both PPARα and PPARγ with high affinity in mice (PPARα EC_{50} = 0.6μM; PPARγ EC_{50} = 10.1 μM) [52-54]. PPARα and PPARγ are major regulators of lipid metabolism and adipocyte differentiation respectively [55]. *In vivo* studies have revealed treatment with MEHP results in activation of enzymes downstream of PPARα activation in rodents as well as promoting obesity and adipocyte differentiation in mice [56, 57].

Human exposure to DEHP

Because DEHP is not covalently bound to the plastic polymer, it is able to leach out into the environment. This, combined with its widespread usage and contamination leads to lifelong human exposure. Estimation of human exposure is based on indirect measurements of DEHP

metabolites in the urine. In addition to being a non-invasive method of measurement, urine analysis has been shown to be reproducible and predictive of long-term exposures [58, 59]. Daily exposure of the general population to DEHP is estimated to range anywhere between 0.4 to 52.9 μg/kg body weight (BW)/day with some sources predicting values as high as 409 μg/kg BW/day [60-62]. The major route of exposure to DEHP in the general population is thought to be ingestion of food products and makes up anywhere between 4.9 to 18μg/kg BW/day of the daily estimated intake [63]. Additional routes of exposure include dermal absorption, inhalation, and parenteral exposure during medical interventions [64, 65].

Interestingly, patients undergoing medical procedures account for some of the highest exposures to DEHP. Procedures involving high exposures of DEHP include parenteral nutrition (0.03 – 0.13 mg/kg BW/day) [66], blood transfusion (1.3 – 2.6 mg/kg BW/day) [67], cardiopulmonary bypass and extracorporeal membrane oxygenation (0.3 – 2.4 mg/kg BW/day) [66], and hemodialysis (0.02 – 0.36 mg/kg BW/day) [68]. Of particular concern are exposures to neonates and young children. Studies of infants in the NICU revealed exposure levels that were several-fold higher when compared to children >6 years of age in the general population [69, 70]. Exchange transfusions are thought to deliver the highest dose of DEHP (up to 22.6 mg/kg BW/day) to newborn infants [67, 71].

Occupational exposure to DEHP is another cause for concern amongst factory workers. One study reported DEHP levels ranging from 0.02 – 4.11 mg/m^3 in the air resulting in a two- to ten-fold increase in urine DEHP concentrations following their shift [72, 73]. The Agency for Toxic Substances and Disease Registry estimates an occupational exposure level of 143 – 286 μg/kg BW/day [74] while the European Chemicals Agency provides a higher estimate of 1 – 2 mg/kg BW/day [75].

Fetal and neonatal exposure to DEHP occurs as a consequence of pregnant and lactating women being exposed to phthalates in every day life. DEHP has been detected in umbilical cord blood [76, 77] as well as amniotic fluid [78, 79]. Furthermore, DEHP is found in both breast milk and infant formula suggesting prolonged DEHP exposure begins early in fetal life and has the potential to affect development [80, 81]. The dose delivered to infants via breast milk is though to range between 0.008 – 0.021 mg/kg BW/day but has the potential to be manifold higher for women undergoing hemodialysis [66].

Pharmacokinetics of DEHP

DEHP is comprised of a phthalic acid group and two branched alkyl chains which results in the generation of multiple metabolites within the body (Figure 3). Upon absorption, DEHP undergoes an initial hydrolysis by lipases and esterases in the intestine. This phase I detoxification results in the production of MEHP [65, 82]. Interestingly, MEHP, like many other phthalate monoesters, is the bioactive metabolite and has been found to be ten times more potent *in vitro* than its parent compound, DEHP [83]. MEHP is further converted into more than 10 additional metabolites including 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP, and 2cx-MMHP. Following phase II conjugation, these metabolites are easily excreted in urine and can be measured to assess DEHP exposure [65, 82].

A single 48.1mg administration of deuterium-labelled DEHP in a human subject resulted in excretion of 70.5% of the initial dose in urine after 24 hours as measured by 5 major metabolites; MEHP, 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP, and 2cx-MMHP. A further 3.8% was excreted in the next 20 hours [84, 85]. In serum, the major metabolite present was MEHP which reached peak concentration (4.95mg/l) 2 hours following administration of DEHP. MEHP also reached

its peak concentration (3.63mg/l) in urine 2 hours following administration but was, in contrast, the 3rd most abundant metabolite behind 5OH-MEHP and 5oxo-MEHP. A single oral administration of 40, 200, or 1000 mg/kg of ^{14}C labelled DEHP in Sprague-Dawley rats revealed peak concentrations of MEHP in serum and urine 24 hours following exposure. MEHP was almost completely cleared from serum and urine 48 hours following oral administration [86].

Effect of DEHP on human development and reproduction

Retrospective studies of reproductive health in the male has revealed an increasing number of issues including decreased sperm quality as well as an increased rate of cryptorchidism, hypospadias, and testicular cancer [87-90]. These defects are grouped together as the testicular dysgenesis syndrome (TDS) which is thought to have, in part, environmental origins. As such, there is an increasing number of studies identifying an association of DEHP exposure with components of the TDS in both neonate and adult populations [91-94]. Prenatal measurement of three different DEHP metabolites in urine samples of pregnant mothers revealed an inverse correlation with anogenital distance (AGD) in male infants [91, 93]. Measurement of the AGD can be used to indirectly assess fetal androgen levels [95] and can predict semen quality and reproductive ability in the adult male [96, 97]. Furthermore, a decreased AGD is associated with *in utero* exposure to endocrine disruptors, including DEHP [91, 98]. Other endpoints affected by DEHP exposure included an increased rate of cryptorchidism and decreased penile width, both of which are associated with a decreased AGD [98, 99].

Studies on the impact of DEHP on adult reproductive health reveal similar detrimental effects. In males, increased urinary levels of MEHP were associated with both sperm DNA damage and a decrease in steroid hormone levels [92, 94]. The same group found an association

with urinary levels of DEHP metabolites and pre-term births in a cohort of women in Mexico [100]. Similar findings were reported in a cohort of mothers from Italy [77].

Effect of fetal exposure to DEHP on rat reproduction

A number of studies in the rat animal model have demonstrated that DEHP exposure, especially during fetal development, has detrimental effects on male reproduction. The fetal period from around GD14 to birth is of particular interest for phthalate exposure as it is marked by the development of key endocrine organs, the adrenal glands and testes (in males), as well as the first peak in testosterone [101]. Treatment of pregnant Sprague-Dawley dams with 750 mg DEHP/kg BW/day from GD14 to PND3 resulted in a reduction of both fetal and neonatal testosterone levels [102]. Furthermore, this *in utero* exposure resulted in malformations comparable to those seen in TDS such as hypospadias and cryptorchidism as well as other abnormalities including decreased AGD, decreased testes weight, and nipple retention [103].

Organ culture of GD20 testes exposed *in utero* to DEHP revealed a dose-dependent reduction in testosterone production beginning at the 117 mg DEHP/kg BW/day dose [104]. hCG-stimulation of these testes in culture resulted in testosterone production unaffected by DEHP exposure suggesting endocrine disruption of the testes in the animal model is limited to basal steroidogenesis. Gene expression analysis of testes exposed *in utero* to DEHP revealed an increase in *Cyp11a1*, *Cyp17a1*, *Star*, and *Tspo* mRNA levels suggesting the steroidogenic pathway was not at fault for testosterone reduction.

Effect of fetal exposure to DEHP on the adrenal glands

Recent studies of DEHP exposure in the Sprague-Dawley animal model have demonstrated that the endocrine disruption of the testes and subsequent reduction in testosterone may be, in part, due to changes in adrenal gland function. Leydig cells have been previously identified *in vitro* to express MR and aldosterone binding results in an increase of both basal, and leutenizing hormone-stimulated testosterone levels [105]. Analysis of PND60 testes exposed *in utero* to DEHP revealed a decrease of MR mRNA and protein levels beginning at the 100 mg DEHP/kg BW/day dose [106]. Serum aldosterone was also found to be reduced by 50% beginning at the same doses [107]. Serum levels of the aldosterone secretagogues, potassium and ATII, were unaffected in these animals. Gene expression analysis of DEHP-exposed adrenals showed no change in the mRNA levels of key steroidogenic proteins and enzymes, including *Star*, *Tspo*, *Cyp11a1*, and *3β-HSD* at the 100 mg dose. Interestingly, an increase in expression of *Hmgcr* and *Hmgcs1* as well as an accumulation of lipid droplets in the zona glomerulosa was seen. A global transcriptomic study of the DEHP-exposed adrenal identified the PPAR and mitogen-activated protein kinase (MAPK) signaling pathways to be affected long-term [108].

Recent epigenomic work has studied the effect of DEHP over a range of doses and identified hotspots of DNA methylation, specifically within CpG islands (40%) and shore/shelf (30%) regions [109]. Interestingly, many loci were found to be differentially methylated even at the dose of 1mg DEHP/kg BW/day, suggesting that the effect of DEHP on the adrenal glands occurs even at doses where aldosterone is unaffected.

Figures 1 to 3

Figure 1. Cholesterol transformation

Sources of cholesterol for steroid biosynthesis include *de novo* synthesis, intracellular lipid droplets, and extracellular lipoproteins. Cholesterol is first moved into the mitochondria where it is cleaved into pregnenolone by CYP11A1. Pregnenolone diffuses out of the mitochondria and moves to microsomes or the endoplasmic reticulum to be converted into testosterone and estradiol in the gonads, and cortisol and aldosterone in the adrenal glands.

Figure 1. Cholesterol transformation

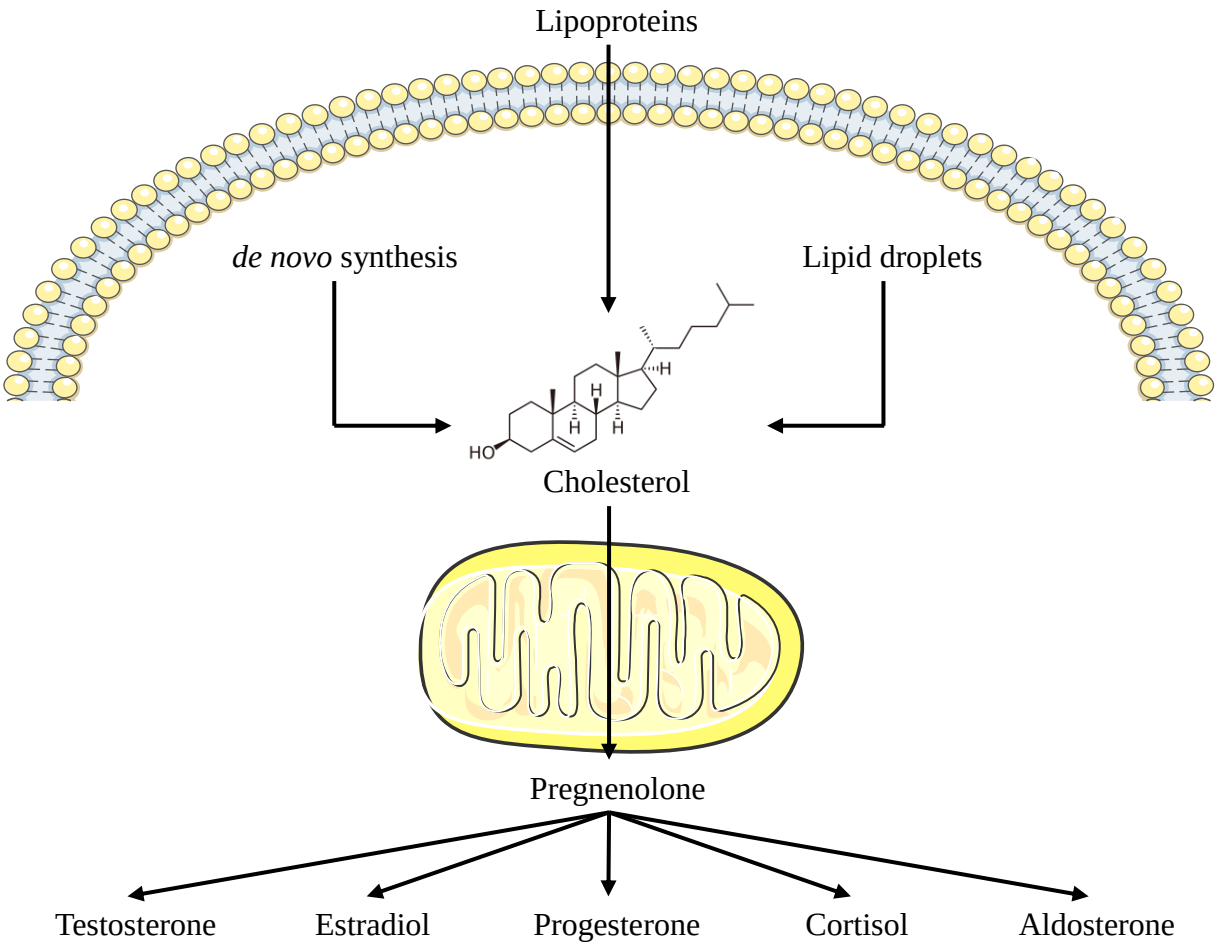


Figure 2. Sequence of events in the renin-angiotensin-aldosterone system

1. Angiotensinogen is constitutively produced by the liver and released into circulation. 2. Upon a decrease in blood volume, the enzyme renin is released by the kidneys. 3. Renin cleaves angiotensinogen resulting in angiotensin I. 4. Angiotensin I is further cleaved into angiotensin II by the angiotensin-converting enzyme (ACE) in the lungs. 5. Angiotensin II acts on the adrenal glands as a secretagogue of aldosterone.

Figure 2. Sequence of events in the renin-angiotensin-aldosterone system

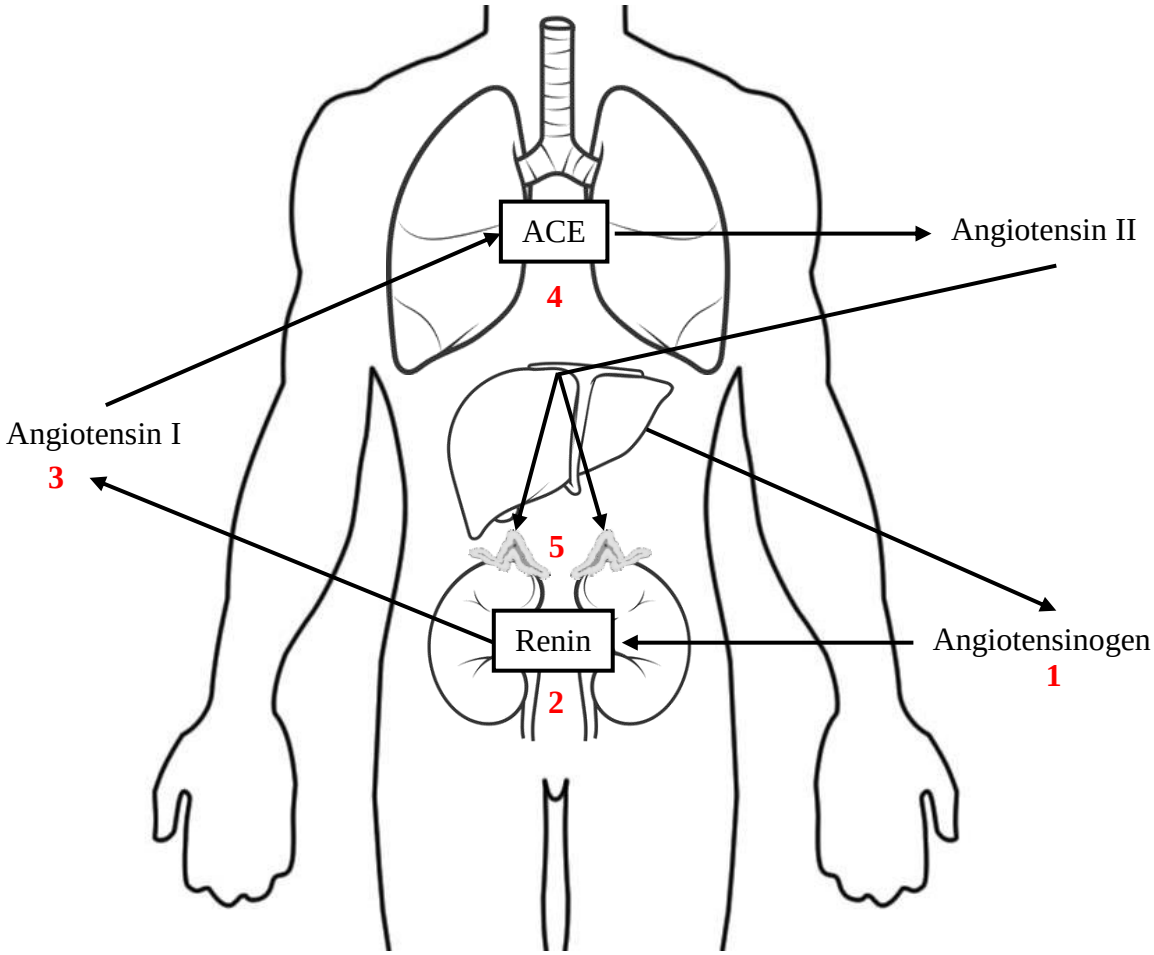
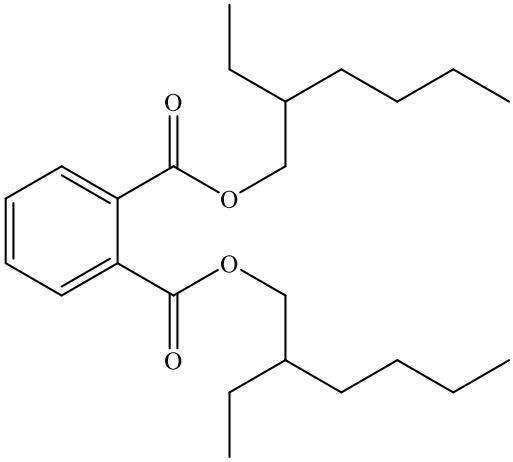


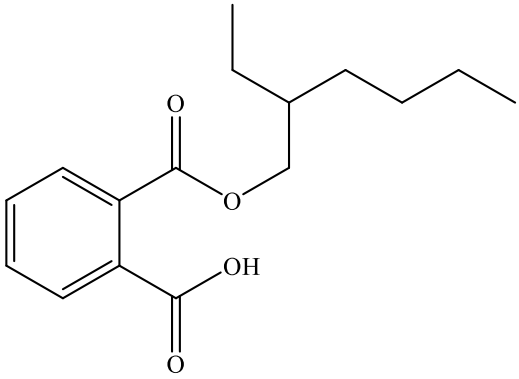
Figure 3. Di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate

Upon absorption, DEHP undergoes hydrolysis by lipases in the intestine resulting in the production of MEHP. MEHP is the bioactive metabolite of DEHP. Following phase II conjugation, MEHP and other metabolites of DEHP are excreted through the urine.

Figure 3. Di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate



DEHP



MEHP

Fetal Exposure to Low Levels of the Plasticizer DEHP Predisposes the Adult Male Sprague-Dawley Adrenal Gland for Endocrine Disruption

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Abstract

In utero exposure to endocrine disrupting chemicals (EDCs) may affect adult health. Di-(2-ethylhexyl) phthalate (DEHP) is an EDC that is widely used in the production of polyvinyl chloride products and a ubiquitous environmental contaminant. Previously, we used a rat model system to show that fetal exposure to DEHP decreased levels of major steroid hormones in adulthood and that environmentally-relevant levels of DEHP affected both gene expression and the same epigenomic loci that were affected by exposure to high levels. We hypothesized that low levels of DEHP exposure insult the endocrine system (a "first hit") and increase its susceptibility to later exposure ("second hit") and subsequent disease. Here, we use the same model to identify pathways affected by low levels of DEHP exposure that are likely to lead to endocrine disruption. We demonstrate that a second hit after fetal exposure affected serum aldosterone. To unveil the first hit influence of early DEHP exposure, we treated *in utero* DEHP-exposed adult offspring with a stressor that targeted the peroxisome proliferator-activated receptor (PPAR) or cholesterol biosynthesis pathways. Treatment with the PPARγ antagonist T0070907 reduced serum aldosterone compared to animals not exposed to DEHP *in utero*. Analysis of gene expression in animals that were subjected to both early and late exposure revealed deregulation of genes for the potassium channel *Kcnk5* and the retinoid-X receptors (RXR) *Rxrα* and *Rxrβ,* indicating that these entities are linked to endocrine disruption. We propose that early exposure to environmental doses of DEHP predisposes the animal for disease later in life.

Introduction

Endocrine disruptors (EDCs) are chemicals that interfere with endogenous endocrine signaling [110]. Man-made EDCs are present in a wide variety of commercial products and are ubiquitous environmental contaminants. This has led to widespread exposure of humans to EDCs, as is apparent from studies of their presence in bodily fluids [65, 111]. Given this background, the role of these chemicals in disease is clearly relevant to public health. Exposure to EDCs has been correlated with obesity, diabetes, decreased reproduction, cancer, and other developmental diseases [112]. The mechanisms by which EDCs promote disease are complex and likely epigenetic, because physiological changes are not immediately evident even when exposures are acute and exceed those of the general population. Particularly troubling is the possibility that exposure to EDCs during fetal development or childhood may influence disease later in life, a concern recently articulated by the Endocrine Society [112]. Because EDCs interfere with hormonal signals, their disruptive effects may be greatest during this critical developmental period. The hypothesis underlying this concern is known as the fetal origins of disease hypothesis [113]. The possibility also arises that any health risks associated with early exposure may not be mitigated by removing the chemical from the environment.

DEHP is an EDC used in the manufacturing of polyvinyl chloride products, cosmetics, and as an excipient in some medications [114]. It is not permanently bound to the plastic polymer and is eventually released into the environment, where it may enter the food chain. This is a major source of direct exposure [63]. Other sources of direct phthalate exposure are dermal contact and inhalation of contaminated air particles. Indirect exposure during early development may occur when DEHP and its metabolites are transferred from the mother to the fetus or newborn through the fetal-placental unit and breast milk, respectively [76, 115]. Human

exposure normally ranges from 1.7 to 52.1 μg/kg BW/day [60, 61, 116, 117], but can be as high as 10 mg/kg BW/day during medical procedures such as blood transfusions, use of parenteral nutrition, and extracorporeal perfusion [69, 70, 118-120]. Newborns and infants are exposed to higher levels of phthalates because of their increased food intake and metabolism. Exposure starts soon after birth when the infant encounters the environment [121], in breast-feeding, and in baby formula [122]. High levels of DEHP and its metabolites in urine are positively correlated with cryptorchidism [123], decreased anogenital distance [91], and decreased testosterone levels in an adult population [94].

Animal models have been central in research designed to elucidate the mechanisms by which EDCs affect disease processes, both in the short and long term. Such models have permitted researchers to identify the developmental periods of greatest sensitivity to the EDCs ("windows of exposure") and dissect complex epigenetic mechanisms [124]. In previous studies with a rat model system, we exposed pregnant dams to DEHP, beginning on GD14, shortly after the fetal adrenal glands and testes had separated from their adrenogonadal precursor, and continued until the dams gave birth [125, 126]. This *in utero* exposure affected the adult endocrine physiology resulting in a 50% reduction in serum testosterone and aldosterone in adult male offspring [104, 106, 107]. This decrease in aldosterone persisted in the elderly rat and correlated with a lower blood pressure [127] suggesting adrenal function was affected in the long term. In the female offspring, a similar effect was seen with estradiol and aldosterone levels being reduced following *in utero* exposure to DEHP [128].

This research led us to attempt to elucidate the mechanism of the long-term effects of DEHP [124] on adrenal function. Our findings can be summarized as follows: The PPAR and MAPK signaling pathways were affected in the long term by DEHP, as shown by global gene expression

analysis in whole adrenal glands [108]. We have identified that the lipid and lipoprotein metabolism, ATII-, and potassium-stimulation pathways are affected by DEHP in adults but not in the adolescent offspring. We also noted that immune system-related genes are affected by DEHP exposure. Of particular interest is that, while endocrine disruption in male offspring was detected at 100 mg DEHP/kg BW/day, we identified changes in *Ppara* mRNA expression at a much lower dose, 1 mg/kg BW/day, similar to those detected in the environment. It may be, then, that the levels of DEHP in the human environment are sufficient to affect gene expression. In our study, DEHP was cleared from the offspring soon after birth, and they were not exposed to DEHP again. This led us to speculate that adult gene expression changes in response to DEHP are mediated by an epigenetic mechanism. We used a genome-wide screening technique to analyze this question and identified several hotspots of DNA methylation in the adult adrenal gland [109]. Moreover, similar to the mRNA changes in *Ppara* observed at low levels of DEHP, we identified several CpGs that were affected at dosages as low as 1 mg DEHP/kg BW/day. We suggest that low levels of DEHP exposure were sufficient to affect the epigenome and gene expression, but the adrenal gland compensated for these changes to maintain endocrine homeostasis.

We propose that initial exposure to low doses of DEHP during a fetal window of development (a "first hit") may later affect the adrenal gland to increase its vulnerability to a second exposure (a "second hit") that compromises adrenal function. Here, we identify gene changes in key adrenal pathways that we define as the first hit of DEHP. We utilized various compounds that target the PPAR and cholesterol biosynthesis pathways as secondary stressors in adults that had been exposed *in utero* to DEHP. We show that gene changes following prenatal exposure to DEHP predispose the adrenal steroid physiology for a second hit in the adult.

Materials and methods

Animal care and DEHP treatment

Timed pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA). Rats had access to chow (Harlan Laboratories, Indianapolis, IN, USA; Catalog no. T.201815) and water ad libitum and were maintained in a 12-hour light, 12-hour dark cycle with lights on at 7:00 AM. The pregnant dams were treated according to one of four regimens. Pregnant dams were gavaged daily with either corn oil (the vehicle) or DEHP at one of these doses: 0.5, 1, 50, 100, or 300 mg DEHP/kg BW/day (Sigma-Aldrich Corp., St. Louis, MO, USA), from GD14 to parturition (PND 0). We refer to *in utero* exposure of 0.5 and 1 mg DEHP/kg BW/day as low-level exposure and 100 and 300 mg DEHP/kg BW/day as high-level exposure. Male offspring were euthanized with CO_2 on the morning of PND60. Blood was collected by percutaneous cardiac puncture and adrenal glands were snap frozen. Previous work from the laboratory has examined and documented cellular and molecular changes in the *in utero* DEHP exposed gonads, adrenals, and other tissues throughout the development of the male. Although some work has been performed in the female offspring ovaries, females were excluded from this study due to major sex-dependent developmental, physiological, cellular, and molecular differences, including hormone fluctuations throughout the estrus cycle. The animals were handled according to the protocols approved by the McGill University Animal Care and Use Committees.

RNA extraction and global gene expression analysis

Adrenal glands were collected from the offspring on PND60 and the adrenal capsule containing the zona glomerulosa was isolated as previously described [129]. Total RNA was

extracted using the AllPrep DNA/RNA/miRNA Universal kit (Qiagen, Hilden, Germany) that included a DNase step, according to the manufacturers' instructions. The quality and concentration of total RNA were assessed with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA was reverse transcribed, fluorescently tagged, and hybridized to a Rat Gene 2.0 ST Array (Affymetrix, Inc., Santa Clara, CA, USA) at the Genome Quebec Innovation Centre. Data analyses were performed by Dr. Jaroslav Novak (STADIM, Montreal, QC, Canada) as previously described [130]. Briefly, gene signals were processed and normalized using the Robust Multi-array Average (RMA) algorithm. Differential expression of genes compared to the vehicle was analyzed using a t-test with the Benjamini-Hochberg correction for multiple testing complemented by the non-parametric consecutive sampling method. The genes selected in the 0.5 mg DEHP/kg BW/day group satisfied the following conditions: ratio of sample averages ≥ 1.3 with a Benjamini-Hochberg false discovery rate (FDR) value ≤ 0.1. Genes excluded by the Benjamini-Hochberg correction but with a consecutive sampling coincidence number ≥ 10 out of 16 pair-wise comparisons were included for analysis. The upregulated genes selected in the 1 mg DEHP/kg BW/day treatment group satisfied the following condition: ratio of sample averages ≥ 1.3 with a less stringent Benjamini-Hochberg FDR value ≤ 0.15 due to a smaller sample size. The downregulated genes selected had a ratio of sample averages ≥ 1.3 with a Benjamini-Hochberg FDR value ≤ 0.1. Genes excluded by the Benjamini-Hochberg correction but with a consecutive sampling coincidence number ≥ 7 out of 12 pair-wise comparisons were included for analysis. After quality-control analysis, four replicates ($n = 4$) comprised the vehicle group (control) and the group receiving 0.5 mg DEHP/kg BW/day and three replicates ($n = 3$) comprised the group receiving 1.0 mg DEHP/kg

BW/day. Pathway analysis and gene clustering were performed using the DAVID [131] and Reactome [132] bioinformatics portals. Venn diagrams were prepared using FlexArray 1.6. Supplemental Tables 1 and 2 contain a list of the differentially expressed genes. Selected genes were compared to global gene expression data previously obtained from whole adrenal glands exposed *in utero* to 300 mg DEHP/kg BW/day [108]. Gene expression data of primary zona glomerulosa cells treated with 100 nM ATII or 16 mM potassium for 2 hours were retrieved from GEO Datasets with the accession number GSE8421 [133].

Quantitative real-time PCR analysis

Reverse transcription of 800 ng total RNA was performed with the QuantiTect reverse transcription kit (Qiagen) according to the manufacturers' instructions. Quantitative PCR (QPCR) was performed on the LightCycler 480 System (Roche Diagnostics, Basel, Switzerland). The multiplex QPCR mix consisted of 10 µL TaqMan Gene Expression Master Mix (Thermo Fisher Scientific), 2 µL cDNA, 6 µL water, 1 µL FAM-labeled gene target TaqMan probe, and 1 µL VIC-labeled glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) for a final volume of 20 µL. *Gapdh* was used as an endogenous reference gene. The comparative Ct method was used to calculate gene expression relative to the *Gapdh* reference gene. Supplemental Table 1 contains a list of the TaqMan probes used with the TaqMan gene expression master mix (Thermo Fisher Scientific).

Stressor treatment

Adult male offspring of pregnant dams were injected intraperitoneally with vehicle or a stressor compound from PND54 to 59. The stressor compounds were atorvastatin (0.5 mg/kg

BW/day), GW0742 (0.5 mg/kg BW/day), T0070907 (1 mg/kg BW/day), pioglitazone (1 mg/kg BW/day), GW0742 + atorvastatin (0.5 mg/kg BW/day each), T0070907 + atorvastatin (1 and 0.5 mg/kg BW/day respectively), and pioglitazone + atorvastatin (1 and 0.5 mg/kg BW/day respectively) (Tocris Bioscience, Bristol, United Kingdom). Compounds were dissolved in phosphate-buffered saline containing 10% DMSO and 10% Tween-80.

Serum measurements

Serum aldosterone was measured with a monoclonal enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA; Catalog No. 10004377) according to the manufacturer's instructions. Testosterone was measured using radioimmunoassay as previously described [106] with a 18.75% cross-reactivity for 5α-DHT and 0.56% cross-reactivity for androstenedione. The intrassay coefficients of variation for the aldosterone and testosterone immunoassay were 5.5% and 8.2% respectively. All samples were assayed in duplicate. Mean values of serum aldosterone ranged between 64.3 – 900.8 pg/ml and were above the 80% B/B_0 limit. Mean values of serum testosterone ranged between 0.25 – 2.67 ng/ml and were above the 80% B/B_0 limit. Serum was shipped to the Diagnostic Laboratory Services at McGill Comparative Medicine and Animal Resources Centre for measurement of serum electrolytes, cholesterol, high-density lipoproteins, and triglyceride levels.

Statistical analyses

GraphPad Prism program (GraphPad Software, Inc.) was used to calculate the mean ± SEM of the data. Statistical analyses of data were performed by Student's t-test, one-way ANOVA followed by Dunnett's post-hoc tests, or two-way ANOVA followed by Bonferroni's post-hoc

tests. The experimental unit was the pregnant dam. Three to four male offspring, each from different mothers, were examined independently for global gene expression analysis. Five to six male offspring, each from different mothers, were examined independently for QPCR analysis of DEHP-exposed zona glomerulosa. Five to eight male offspring, each from different mothers, were examined independently for serum hormone levels and QPCR analysis in the second hit experiment.

Results

In utero exposure to doses of DEHP similar to those in the environment affect the expression of genes in pathways that are associated with adult endocrine disruption at higher levels of DEHP exposure.

From earlier work, we had ascertained that *in utero* exposure of male rats to high levels of DEHP altered aldosterone secretion and gene expression when the animals reached adulthood [107, 108]. We wished to determine if, and to what extent, low-level exposure (more similar to levels in the environment) affected these same genes. We assessed gene expression with global gene analysis in the zona glomerulosa from adult males that had been exposed *in utero* to 0.5 or 1 mg DEHP/kg BW/day and compared the results with our previous data of males exposed *in utero* to 300 mg DEHP/kg BW/day. The *in utero* exposure to DEHP deregulated 230 and 376 genes in the 0.5 and 1 mg DEHP/kg BW/day treatment groups, respectively, and 103 of these genes were altered in both groups (Fig. 4A). There were 39 genes with altered expression in all three exposure levels, 21 genes with altered expression in both the 1 and 300 mg DEHP/kg BW/day exposures, and 7 genes with altered expression both the 0.5 and 300 mg DEHP/kg BW/day exposures (Fig. 4B).

We wished to identify cellular functions and biological processes that were over-represented and common to the 39 alternatively-expressed genes. Using the DAVID bioinformatics portal [131], we identified several genes that regulate transcription; these were *Creb3l1*, *Crem*, *Giot1*, *Hhex*, *Iqub*, *Irak2*, *Nfil3*, *Nr4a1*, *Nr4a3*, *Ppp1r10*, and *Scx* (shown in red in Fig. 4B), and genes that regulate cholesterol metabolism; these were *Hmgcr*, *Insig1*, and *Ldlr* (shown in blue in Fig. 4B). We also used the Reactome portal [132] to identify genes that are related to the immune system, specifically *Dusp8*, *Irak2*, *Irs2*, *Nr4a1*, *Spsb1*, and *Tuba4a* (underlined in Fig. 4B).

It was also of interest to determine if *in utero* exposure to DEHP affected the ATII or potassium pathways in the adrenal gland of the male rats. This was assessed by comparing gene expression in primary zona glomerulosa cells stimulated with 100 nM of ATII or 16 mM of potassium [133] with gene expression in adrenal glands of the male offspring exposed *in utero* to low doses of DEHP. Global expression arrays were prepared and the results are presented in Figures 4C and 4D. Some of the genes that were differentially expressed in response to ATII or potassium were also differentially expressed in the DEHP-exposed adrenal glands of the male rats.

In utero exposure to low doses of DEHP deregulates zona glomerulosa pathways

In our previous transcriptomic work of whole adrenal glands from males exposed *in utero* to high doses of DEHP, we determined that the PPAR and MAPK signaling pathways are deregulated by DEHP, as well as cholesterol biosynthesis, lipid metabolism, and immune function in the pubertal and adult rat [108, 109]. Since this initial work was performed at high levels of DEHP, we wanted to determine if the genes that were deregulated at the lower doses are also involved in these pathways. Table 1 shows the results of the low-dose exposure. There were several genes deregulated by the low-DEHP doses that are associated with the innate and adaptive immune system and indeed, some of the genes deregulated at low doses are in the same pathways as those affected at the higher doses. These genes are involved in lipid and lipoprotein metabolism, PPARα-activated gene expression, MAPK family signaling, and transcription of nuclear receptors.

We concluded from previous results that *in utero* exposure of male rats to high levels of DEHP altered the expression of several transcription factors in the adrenal gland and these

effects may be long-term. We observed here that low doses of DEHP also affected the gene expression of several transcription factors (Table 2). Some of these, for example *Nr4a1*, are critical for zona glomerulosa function [133]. We interpret these data to support the hypothesis that low levels of DEHP *in utero*, which are insufficient to affect aldosterone levels, deregulate some of the same genes that mediate the adrenal endocrine disruption at higher DEHP doses.

We used QPCR to characterize the expression of selected genes that were deregulated by low and high doses of *in utero* DEHP exposure. *Nr4a1*, *Nr4a3*, *Hmgcr*, *Insig1*, *Ldlr*, and *Kcnk5* had similar expression profiles (Fig. 5). The dose-response curve for expression of each gene was non-monotonic, with increased gene expression levels at 0.5, 1, and 300 mg DEHP/kg BW/day, and with expression levels similar to the control at intermediate doses.

In utero exposure to DEHP predisposes the adrenal gland for endocrine disruption

Based on the results presented, we concluded that genes with altered expression in response to both high and low doses of DEHP share common pathways. We next tested the hypothesis that the changes in gene expression at low doses of DEHP increased the susceptibility of the adrenal gland to further endocrine disruption. We reasoned that if the adrenal glands of animals exposed to DEHP *in utero* were more susceptible than control animals, stressing the gland in adulthood would impair adrenal function of the exposed animals more than the control. We used compounds that target either the PPAR or the cholesterol biosynthesis pathway as the agents of endocrine disruption, two of the pathways most dramatically affected by high levels of DEHP exposure. Male rats that had been exposed *in utero* to low levels of DEHP were injected with the PPARβ agonist GW0742, the PPARγ antagonist T0070907, or the PPARγ agonist pioglitazone, either alone or in combination with atorvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A

(HMG-CoA) reductase inhibitor. Atorvastatin was used to target the cholesterol biosynthesis pathway, a pathway previously shown to be deregulated by exposure to high levels of DEHP [107, 108]. We predicted that by targeting two separate pathways with the second hit, adrenal function might be sufficiently impaired. Serum aldosterone levels at PND60 showed that the PPARγ antagonist, T0070907, decreased aldosterone levels compared to the control (Fig. 6A), but not when atorvastatin was supplied in conjunction with the antagonist. Two-way ANOVA among all stressors tested showed a significant difference with respect to the stressor treatment in aldosterone levels ($p = 0.0044$). Interestingly, two-way ANOVA of all treatment groups, except pioglitazone and pioglitazone plus atorvastatin, showed a significant difference due to DEHP exposure ($p = 0.0294$).

Testosterone levels were unaffected in DEHP-exposed males by any of the stressor combinations shown (Fig 6B). Two-way ANOVA showed a significant difference with respect to the stressor treatment ($p = 0.0030$).

Since T0070907 was the only stressor for which a t-test revealed a significant effect, this stressor was selected for further experiments. We used this compound to further study possible effects of the stressor in animals exposed to DEHP *in utero*. The reduced aldosterone levels observed in DEHP animals did not affect the circulating levels of serum electrolytes (data not shown).

Retinoid-X receptors are involved in the decreased aldosterone levels after T0070907 stressor treatment

We wished to determine whether the PPARs and PPAR-related nuclear receptors were affected by the PPARγ antagonist, T0070907, and if these receptors are involved in reduced

aldosterone secretion. Expression levels of *Ppara* and *Pparg* were not affected by the antagonist (Fig. 7A, B), while *Ppard* levels increased after treatment of the animals with T0070907. *In utero* exposure to 1 mg DEHP/kg BW/day alone resulted in a two-fold increase in *Ppard* expression compared to the DEHP-free control (Fig. 7C). We then measured gene expression of the RXRs. The RXRs heterodimerize with PPARs and bind to PPREs in the DNA to regulate gene expression, making them key components of PPAR function and activity. Two-way ANOVA reveals an interaction between DEHP exposure and T0070907 treatment that resulted in a decrease in *Rxra* and *Rxrb* levels when compared to the DEHP-free control (Fig. 7D, E). There was no significant change in *Rxrg* levels (Fig. 7F) after treatment with the stressor.

The first hit of DEHP involves the Kcnk5 potassium channel

We wished to identify genes that facilitate the decrease in serum aldosterone after exposure to DEHP and treatment with T0070907. To this end, we measured mRNA levels of the ATII receptors and two potassium channels, *Kcnn2* and *Kcnk5*. Adrenal glands were collected from male offspring that had either not been exposed to DEHP *in utero* (one control) or that had been exposed *in utero* to 1 mg DEHP/kg BW/d. Additionally, one group was treated in adulthood with T0070907. The mRNA from the zona glomerulosa was isolated and analyzed with QPCR. *Agtr1a* and *Agtr1b* mRNA levels were less in the glands from the animals treated with T0070907 (Fig. 8A, B) than in those from animals not treated with the stressor, and *Agtr1b* mRNA levels were reduced by approximately 50% in the animals exposed to DEHP alone compared to the ones not treated *in utero*. There were no changes in *Agtr2* levels (Fig. 8C) in any of these treatments.

Kcnn2 and *Kcnk5* encode potassium channels and were deregulated in whole adrenal glands at high levels of DEHP [108]. Messenger RNA levels of *Kcnn2* were less in the animals treated with T0070907 in adulthood (Fig. 8D) than in control animals. Also, there was a trend for less *Kcnn2* mRNA levels in glands from animals treated *in utero* with DEHP alone compared to untreated animals (Fig. 8D). Conversely, there was a two-fold increase in *Kcnk5* mRNA levels in animals exposed *in utero* to DEHP alone (Fig. 8E); T0070907 treatment resulted in a decrease of *Kcnk5* compared to DEHP-free males.

In utero exposure to 1 mg DEHP/kg BW/day and T0070907 stressor treatment does not affect expression of lipid and steroidogenic genes

We previously established that the hormone-sensitive lipase-mediated triglycerol hydrolysis pathway and cholesterol biosynthesis pathway are targets of DEHP in the whole adrenal upon *in utero* exposure to ≥100 mg DEHP/kg BW/d [107, 108]. We measured mRNA levels of *Lipe*, *Plin*, *Prkacb*, *Mgll*, and *Fabp4*, genes associated with lipid metabolism, and *Hmgcr* and *Scarb1*, genes associated with cholesterol metabolism. mRNA levels of *Lipe* in the adrenal glands of male offspring treated with the stressor were lower than those of the untreated controls (Supplemental Fig. 1A). There was no significant effect of DEHP exposure or stressor treatment on *Plin*, *Prkacb*, *Mgll*, or *Fabp4* (Supplemental Fig. 1B-E). mRNA levels of *Hmgcr* in the adrenal glands of stressor-treated male offspring were higher in comparison to untreated animals (Supplemental Fig. 1F). There was no change in mRNA levels of *Scarb1* in the adrenal glands (Supplemental Fig. 1G). Serum analysis revealed that circulating levels of cholesterol, triglycerides, and high-density lipoproteins were unaffected (data not shown).

We explored expression of key steroidogenic enzymes and proteins as a potential explanation for the reduction in serum aldosterone. *Star* mRNA levels were increased in the T0070907 treatment group (Supplemental Fig. 2A). Exposure to 1 mg DEHP/kg BW/day alone resulted in a slight increase in *Star* expression compared to the DEHP-free control. There was no change in *Tspo* levels (Supplemental Fig. 2B). The level of Cyp11b2, the final enzyme in aldosterone biosynthesis, was unchanged by DEHP or the stressor treatment (Supplemental Fig. 2C).

Discussion

We previously showed that fetal exposure to high levels of DEHP affected aldosterone blood serum levels in the adult rat although levels at weaning were normal. We hypothesized that expression of an adrenal gene(s) critical for adult aldosterone biosynthesis was affected in the long term [107] by *in utero* exposure. Since DEHP is rapidly cleared from the body and the offspring were not exposed again, we further hypothesized that the endocrine disruption of the adult adrenal gland was mediated by an epigenetic mechanism that had been altered in the fetus, during development, although how this alteration came about is unknown.

Global gene expression analysis from adrenal glands collected at weaning and in the young adult revealed that high levels of DEHP *in utero* affected the PPAR signaling and MAPK pathways at both time points [108]. However, it was only in the adult offspring that the cholesterol biosynthesis, angiotensin, and potassium pathways were deregulated by these high doses [108]. Deregulation of these particular pathways is strong evidence that DEHP hinders critical pathways in the aldosterone-producing cell: cholesterol biosynthesis provides the building blocks for steroid hormones and the other two pathways are the main regulators of zona glomerulosa function. Although DEHP has been mostly associated with anti-androgenic and reproductive effects [102, 104, 134], these results lead us to suggest that it also affects adrenal gland function.

In the background work to this study, we used a genome-wide DNA methylation technique to characterize the long-term epigenetic changes [135]. We showed that DEHP induced changes in CpG methylation levels throughout the adrenal epigenome and that some of the differential DNA methylation was clustered into hotspots in the genome. Interestingly, we observed changes in expression of *Pparα* and the DNA methylation of some CpG loci at low doses of DEHP that

were not reflected in alteration of adrenal function and aldosterone secretion in the adult. These data led us to hypothesize that exposure to low doses of DEHP acts to increase the susceptibility of the genome to further exposure. We refer to this as the "two-hit" hypothesis.

While our previous experiments were carried out in whole adrenal glands, the present work was done with purified zona glomerulosa cells in the hope of enhancing the sensitivity and specificity of detection methods. We also used a low dose of DEHP (1 mg/kg BW/day), which corresponds to the human equivalent of 0.16 mg DEHP/kg BW/day. Although higher than the estimated environmental human exposures of 13.8 μg DEHP/kg BW/day [60], it is within the range of medical [50, 66] and occupational [74, 75] exposures in adults (0.005–8.5 mg and 0.143–2 mg DEHP/kg BW/day, respectively).

Our first venture in this study was to characterize the global gene expression in the adult zona glomerulosa caused by fetal exposure to 0.5 and 1 mg DEHP/kg BW/day. There were 103 genes differentially expressed relative to the control and present in both treatments. We conclude that DEHP affected gene expression in the zona glomerulosa at doses two orders of magnitude lower than those required for endocrine disruption.

To compare the effects of a low and high exposure to DEHP, we sorted the gene changes into the pathways we previously identified to be deregulated by high exposure to DEHP using the Reactome bioinformatics portal. The data show that genes in many of the pathways associated with endocrine disruption at high doses were deregulated at the lower doses of exposure. Genes in lipid-related pathways, which include cholesterol biosynthesis, the PPAR and MAPK pathways, and the nuclear receptor transcription pathway, were affected. Of interest, pathways related to immune function that include genes involved in the innate and adaptive immune system and in cytokine signaling were particularly affected by low doses of DEHP.

These data are in agreement with our previous epigenetic study, which showed two hotspots of DNA methylation and gene deregulation following DEHP exposure in chr20p12 [135], which houses the genes controlling the immune response [136]. Moreover, we and others have reported that DEHP also affects the immune system in adipose tissue [137] and testes [138] and may be caused by a variety of EDCs [139]. Together, the immune-related changes suggest that the immune response is also a target of DEHP and that the distinct effect of DEHP on chr20p12 may be mediating other diseases associated with DEHP exposure, such as asthma [140], atopic disorders [141, 142], and macrophage dysfunction [143, 144]. The data also showed that genes critical to the core zona glomerulosa function were targeted by DEHP. *Hmgcr*, the enzyme involved in the rate-limiting step in cholesterol biosynthesis, was upregulated at both 0.5 and 1 mg DEHP/kg BW/day. This suggested that cholesterol biosynthesis, an important pathway in providing precursors for aldosterone biosynthesis [145, 146], is one of the most sensitive targets of DEHP. Moreover, *Nr4a1*, a master regulator of zona glomerulosa function [133] was also affected in a manner similar to *Hmgcr*. Whether *Nr4a1* and *Hmgcr* were upregulated to counter a drop in aldosterone biosynthesis or whether these genes are directly targeted by DEHP remains to be determined. The gene analysis also showed that *Nr5a1* and *Nr0b1*, genes critical for adrenal development and steroid function [124] and, when mutated, can lead to adrenal insufficiency [147], were also affected by DEHP. These genes, along with *Nr4a1,* are vital for adrenal gland zonation [12, 148]. Based on these data, we conclude that low doses of DEHP during fetal development alter genes critical for adrenal function. While the zona glomerulosa appears to be unable to overcome the disruption of high concentrations of DEHP, it can overcome the endocrine disruption at low doses.

We used Venn diagrams to examine the relationships between the genes affected by DEHP at low and high doses of exposure and those involved in aldosterone biosynthesis after ATII or potassium stimulation. We found 39 genes that were altered in both the low and high DEHP exposures, most of which were upregulated. Pathway analysis showed that these genes are involved in transcription, cholesterol metabolism, and embryonic organ development. It is interesting to note that the potassium channel *Kcnk5* is a common target of DEHP in all these situations. *Kcnk5* expression was inversely correlated, in both dose and time, with the decreased aldosterone levels induced by DEHP [108]. Moreover, we expanded our analysis to include genes that were differentially expressed in the zona glomerulosa stimulated with potassium or ATII. The data indicated that low levels of DEHP also affected the expression of some of these genes. This was surprising since, under normal conditions, the genes identified are only upregulated during acute stimulation. It may be, then, that DEHP alters the pathways that directly control aldosterone biosynthesis. We interpret these data, in total, to mean that low doses of DEHP targeted pathways that are critical and specific to zona glomerulosa function. We hypothesized that some these genes are part of a compensatory mechanism to maintain aldosterone homeostasis and that a second stimulus directed at these pathways will unveil the epigenetic weakness established during fetal exposure to DEHP.

We quantified gene expression changes across the range of DEHP exposure. We observed a non-monotonic response in the expression of the transcription factors *Nr4a1* and *Nr4a3;* in genes related with cholesterol metabolism, including *Hmgcr*, *Insig1*, and *Ldlr*; and in the potassium channel *Kcnk5*. The possibility that this pattern could be accounted for by variations in the reference gene used for the quantification analysis was ruled out by the expression patterns of other adrenal genes (data not shown). This distinct expression pattern, which was also observed

for genes associated with the EDC phenotype, suggests that a common upstream element may be partly mediating the response to DEHP. At present, this upstream element is unknown but we do not rule out that this non-monotonic pattern of expression originated in the epigenomic regions that we previously identified [135]. Interestingly, our previous genome-wide DNA methylation analysis showed that none of the differentially-methylated CpG loci were within or near genes affected by DEHP. Instead, most of the CpG changes occurred in CpG islands, shore, and shelf regions, which are associated with control of gene expression. Moreover, in some cases, the CpG clusters correlated with regional changes in gene expression. This latter point suggested to us that these areas contain regulatory elements that can affect the expression of a cluster of genes.

The next experiment was designed to test the hypothesis that *in utero* exposure to DEHP compromises the adrenal gland, resulting in increased susceptibility to stress in the adult. From previous work, we knew that the PPAR pathway, a mediator of metabolism and immunity, is sensitive to DEHP, so that compounds that affect PPAR nuclear receptors are likely candidates for stressors. The involvement of the PPAR pathway in aldosterone biosynthesis was previously shown *in vitro* in the H295R and HAC15 cell lines [149, 150] and *in vivo*, when a 7-day treatment with rosiglitazone, a PPARγ agonist, reduced serum aldosterone levels in Sprague-Dawley rats [151]. We also considered compounds that affect the rate-limiting step of cholesterol biosynthesis. This information led us to select the PPARβ agonist GW0742, the PPARγ agonist pioglitazone, and the PPARγ antagonist T0070907 as the stressors for this experiment. The stressors were used alone and in combination with atorvastatin, an HMG-CoA reductase inhibitor. These stressors were injected into adult rats that had been exposed to low doses of DEHP *in utero* and aldosterone levels were assessed and compared to animals that had no exposure to DEHP. A two-way analysis of all treatment groups with the exception of

pioglitazone and pioglitazone + atorvastatin revealed DEHP had a significant effect on variation. In particular, T0070907 treatment resulted in a significant reduction of serum aldosterone levels in DEHP-exposed offspring when compared to the oil control. This is a decrease of two orders of magnitude in the threshold for endocrine disruption.

In the last phase of the study, we searched for the changes associated with the second hit, which was supplied with the challenge with T0070907. We examined genes known to be associated with the DEHP phenotype at high doses and assessed their expression in rats receiving low doses *in utero* and that were stressed as adults. We chose to examine PPAR nuclear receptors and the RXRs, as these are known to form dimers with each other. *Rxra* and *Rxrb* expression was influenced by *in utero* exposure to a low dose of DEHP, a result that was unexpected and led us to wonder if the dimerizing partners of the PPARs may be important for endocrine disruption. In addition, gene expression of the ATII receptors and the members of the potassium pathway *Kcnk5* and *Kcnn2* were analyzed. Interestingly, *Rxra*, *Rxrb*, and *Kcnk5* were downregulated with respect to the control and it may be that a common transcription factor controlling their gene expression was absent. *Kcnk5* (also known as TASK-2) encodes an acid-dependent potassium channel and has been shown to play a role in aldosterone production. Specifically, decreased expression of *Kcnk5* has been determined to be a hallmark of aldosterone-producing adenomas [152, 153]. Because other genes involved in lipid metabolism and steroid biosynthesis were unaffected by the DEHP exposure, the data suggest that the RXRs and *Kcnk5* mediated the decrease of aldosterone levels after the second hit.

In summary, the results presented here, in combination with our previous epigenetic and gene expression studies, suggest that *in utero* exposure to environmentally-relevant doses of DEHP predisposes the male adrenal gland for long-term endocrine disruption. Epigenetic

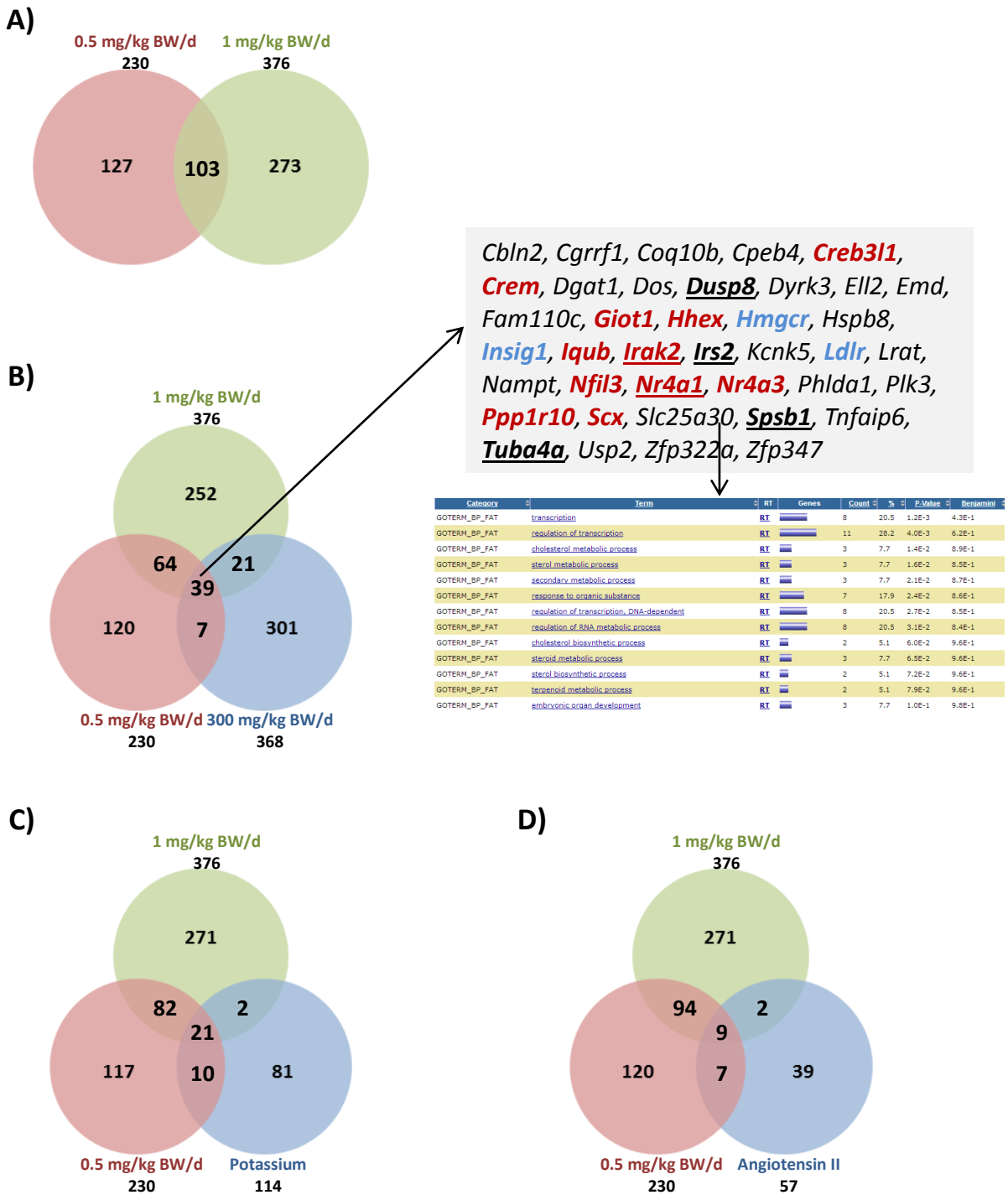
changes caused by low doses of DEHP may result in permanent changes to expression of genes and may be associated with other disease states.

Figures 4 to 8

Figure 4. Venn diagrams of the differentially-expressed genes in the zona glomerulosa of adult rats that were exposed to DEHP

(A) A comparison of the two low doses of DEHP; (B) a comparison of the low doses with the highest dose. Includes an expanded list of the 39 differentially-expressed genes amongst all 3 doses and the over-represented biological processes (BP) obtained from David Bioinformatics. (C) A comparison of the lowest doses of DEHP and the zona glomerulosa stimulated with potassium; (D) a comparison of the lowest doses and the zona glomerulosa stimulated with angiotensin II.

Figure 4. Venn diagrams of the differentially-expressed genes in the zona glomerulosa of adult rats that were exposed to DEHP



Category	Term	RT	Genes	Count	%	P_Value	Benjamini
GOTERM_BP_FAT	transcription	RT		8	20.5	1.2E-3	4.3E-1
GOTERM_BP_FAT	regulation of transcription	RT		11	28.2	4.0E-3	6.2E-1
GOTERM_BP_FAT	cholesterol metabolic process	RT		3	7.7	1.4E-2	8.9E-1
GOTERM_BP_FAT	sterol metabolic process	RT		3	7.7	1.6E-2	8.5E-1
GOTERM_BP_FAT	secondary metabolic process	RT		3	7.7	2.1E-2	8.7E-1
GOTERM_BP_FAT	response to organic substance	RT		7	17.9	2.4E-2	8.6E-1
GOTERM_BP_FAT	regulation of transcription, DNA-dependent	RT		8	20.5	2.7E-2	8.5E-1
GOTERM_BP_FAT	regulation of RNA metabolic process	RT		8	20.5	3.1E-2	8.4E-1
GOTERM_BP_FAT	cholesterol biosynthetic process	RT		2	5.1	6.0E-2	9.6E-1
GOTERM_BP_FAT	steroid metabolic process	RT		3	7.7	6.5E-2	9.6E-1
GOTERM_BP_FAT	sterol biosynthetic process	RT		2	5.1	7.2E-2	9.6E-1
GOTERM_BP_FAT	terpenoid metabolic process	RT		2	5.1	7.9E-2	9.6E-1
GOTERM_BP_FAT	embryonic organ development	RT		3	7.7	1.0E-1	9.8E-1

Figure 5. Histograms showing the gene expression levels in the adult zona glomerulosa of select genes in response to in utero exposure to different concentrations of DEHP

Relative gene expression for (A) *Nr4a1*, (B) *Nr4a3*, (C) *Hmgcr*, (D) *Insig1*, (E) *Ldlr*, *and* (F) *Kcnk5*. Note the non-monotonic dose-response. Data shows control (in white) and DEHP (in black) represented as means ± SEM; n = 5; bars indicate significance by 1-way ANOVA followed by Dunnett's post-test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure 5. Histograms showing the gene expression levels in the adult zona glomerulosa of select genes in response to in utero exposure to different concentrations of DEHP

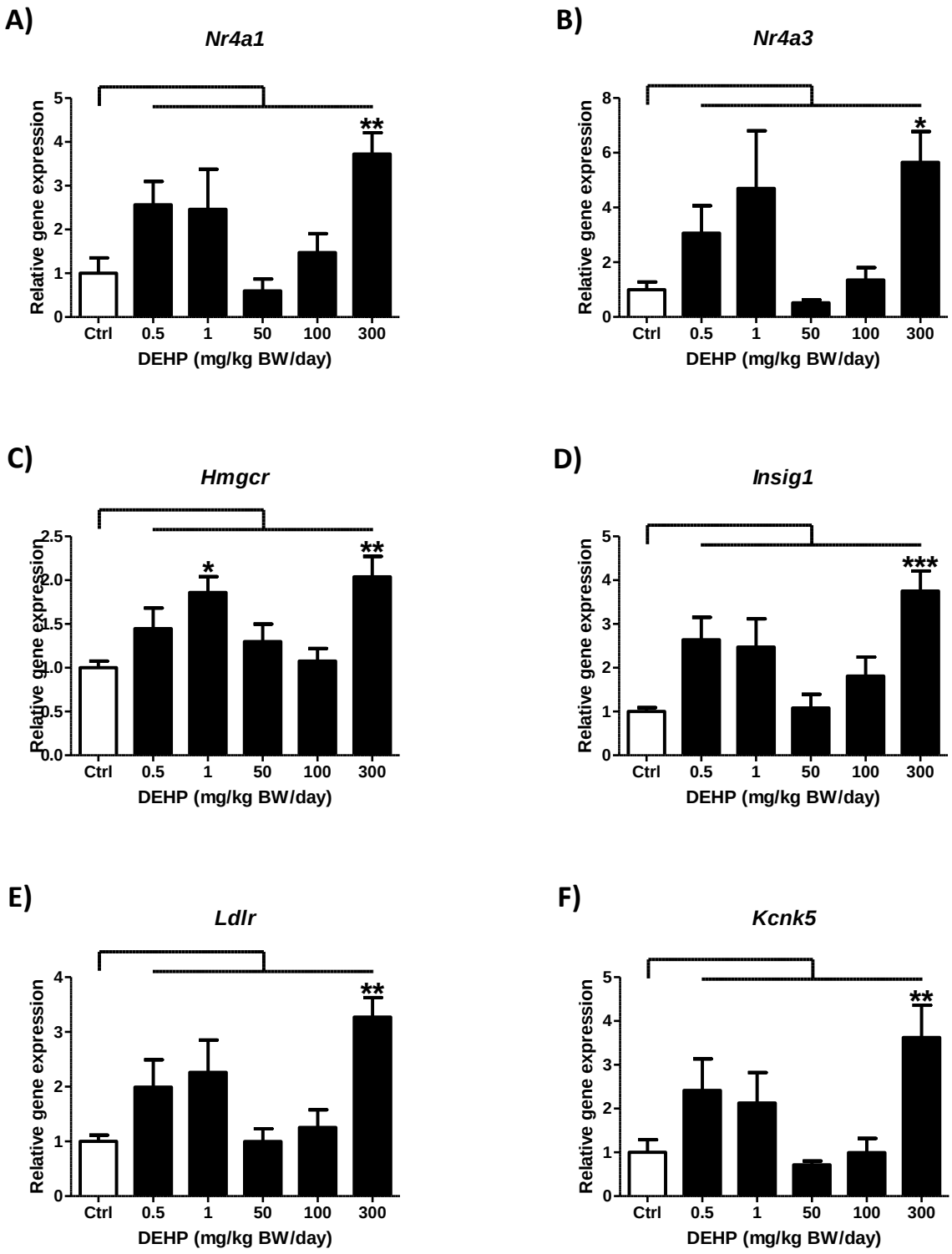


Figure 6. Circulating levels of aldosterone and testosterone in adult male rats treated in utero with DEHP and as adults with secondary stressors, with or without atorvastatin

Serum levels for (A) aldosterone and (B) testosterone are shown. Control animals were not exposed *in utero* to DEHP. Data represent means ± SEM; n ≥ 5; bars indicate significance by Student's T-test or 2-way ANOVA, $*p < 0.05$. Variation caused by DEHP exposure indicated by ϕ, $\phi p < 0.05$. Variation caused by stressor treatment indicated by θ, $\theta\theta p < 0.01$.

Figure 6. Circulating levels of aldosterone and testosterone in adult male rats treated in utero with DEHP and as adults with secondary stressors, with or without atorvastatin

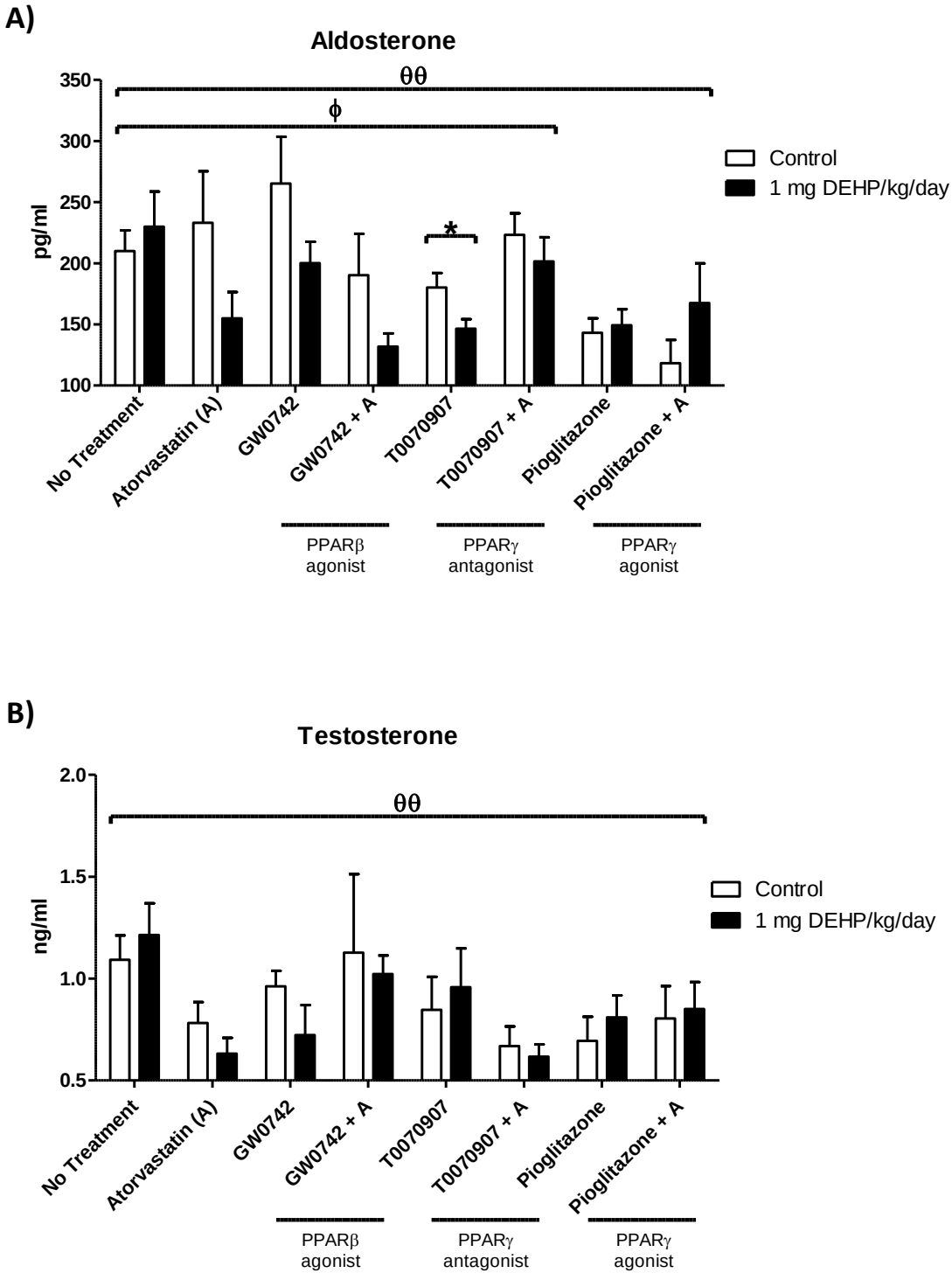


Figure 7. Relative expression of Pparα, Pparγ, Pparδ, Rxrα, Rxrβ, and Rxrγ in the zona glomerulosa of adult male rats treated in utero with DEHP and as adults with T0070907

Gene expression shown for (A) *Pparα*, (B) *Pparγ*, (C) *Pparδ*, (D) *Rxrα*, (E) *Rxrβ*, and (F) *Rxrγ*. Data shows control (C, in white) and 1 mg DEHP/kg BW/day (D, in black) represented as means ± SEM; n ≥ 5; bars indicate significance by Student's T-test or 2-way ANOVA, *$p < 0.05$; ***$p < 0.001$. Variation caused by interaction of DEHP exposure and stressor treatment indicated by #, #$p < 0.05$.

Figure 7. Relative expression of Pparα, Pparγ, Pparδ, Rxrα, Rxrβ, and Rxrγ in the zona glomerulosa of adult male rats treated in utero with DEHP and as adults with T0070907

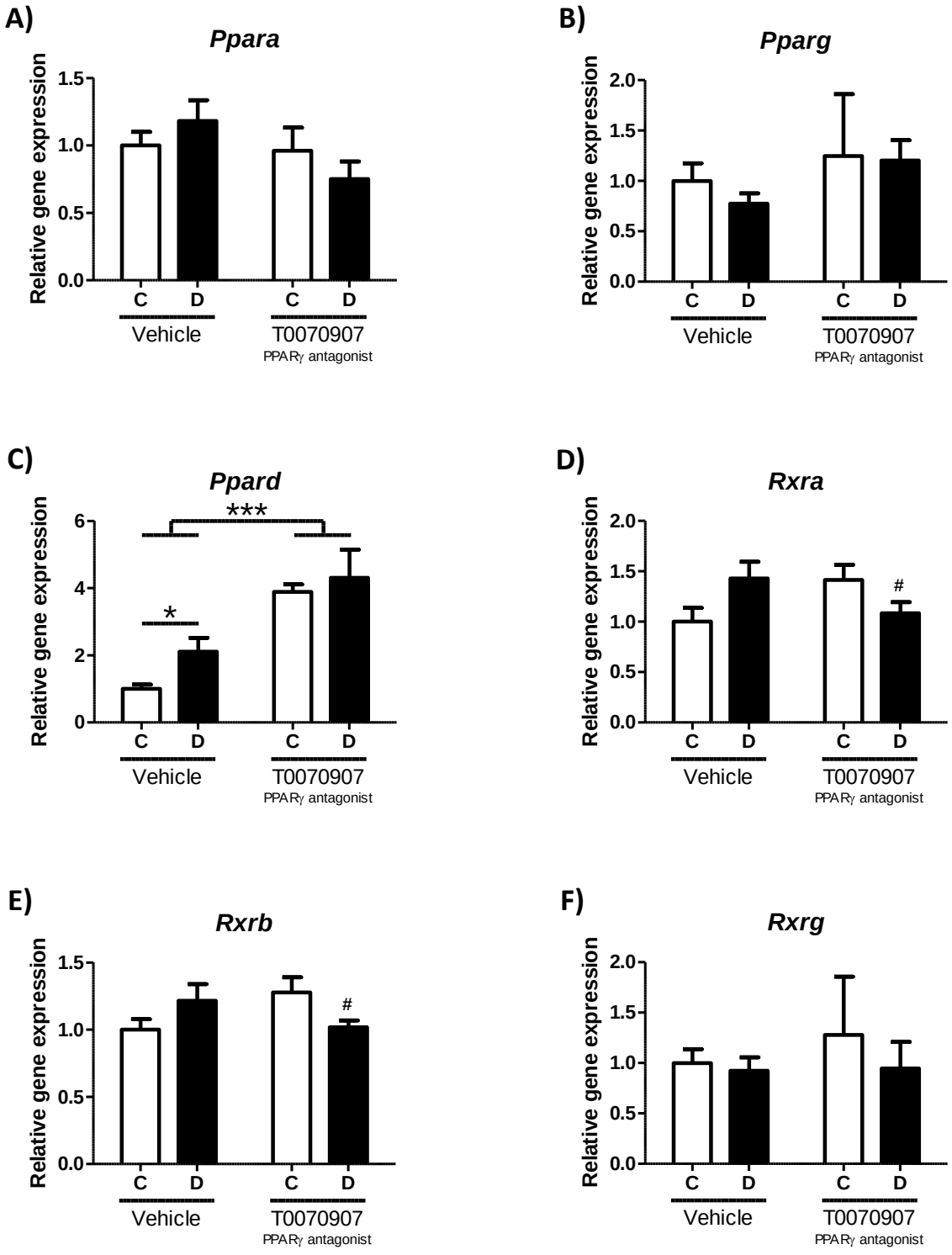
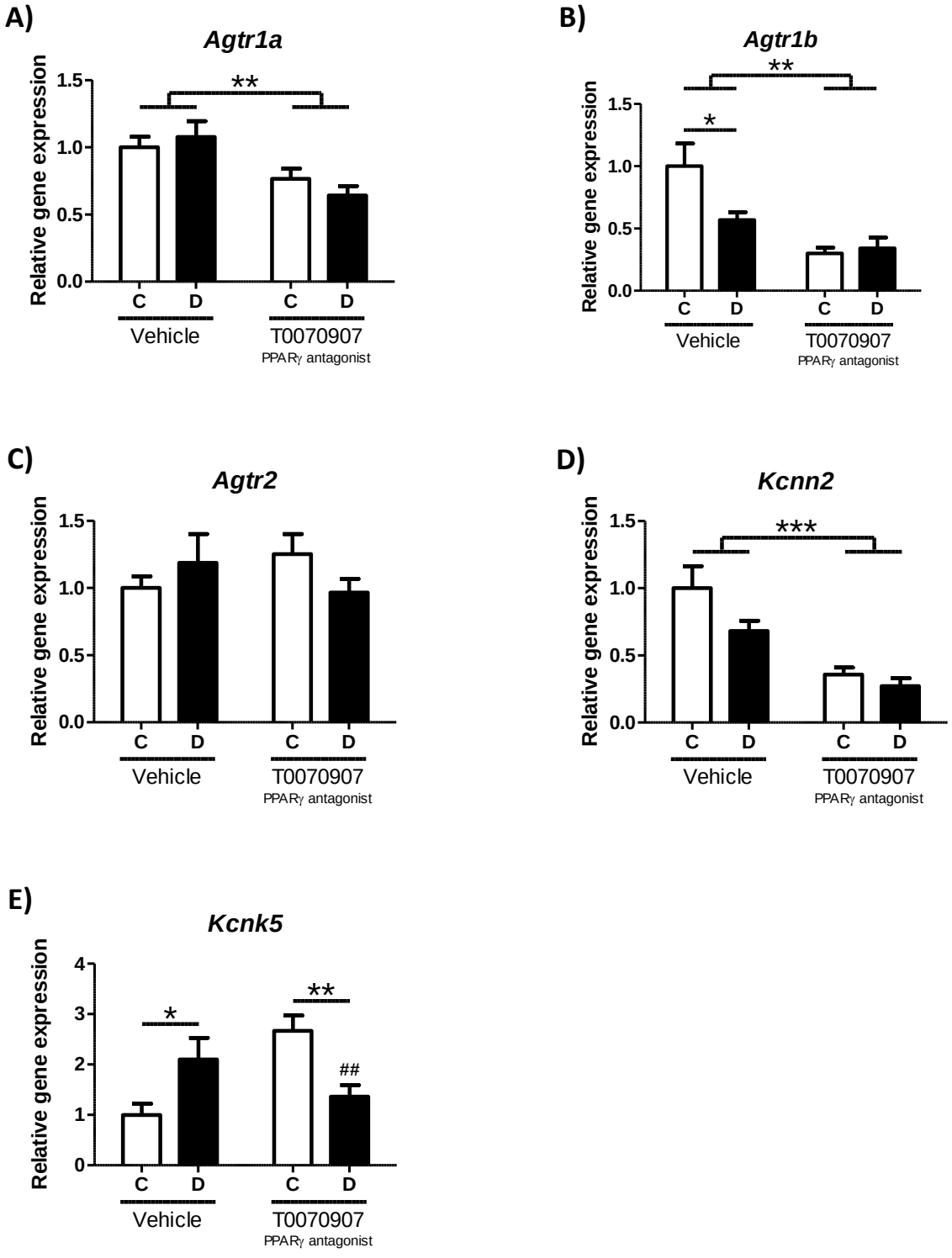


Figure 8. Relative expression of Agtr1a, Agtr1b, Agtr2, Kcnn2, and Kcnk5 of adult male rats treated in utero with DEHP and as adults with T0070907

Gene expression shown for (A) *Agtr1a,* (B) *Agtr1b,* (C) *Agtr2,* (D) *Kcnn2,* and (E) *Kcnk5*. Data shows control (C, in white) and 1 mg DEHP/kg BW/day (D, in black) represented as means ± SEM; n ≥ 5; bars indicate significance by Student's T-test or 2-way ANOVA, **p* < 0.05; ***p* < 0.01; ****p* < 0.001. Variation caused by interaction of DEHP exposure and stressor treatment indicated by #, ##*p* < 0.01.

Figure 8. Relative expression of Agtr1a, Agtr1b, Agtr2, Kcnn2, and Kcnk5 of adult male rats treated in utero with DEHP and as adults with T0070907



Tables 1 and 2

Table 1. Pathways associated with gene changes following exposure to 0.5 or 1.0 mg DEHP/kg BW/day. Downregulated genes in bold.

Pathway	0.5 mg DEHP/kg BW/day	1 mg DEHP/kg BW/day
Innate immune system	*Birc2, Dusp8, Dusp10, Gab2, Irak2, Irf7, Irs2, Jun, Nr4a1, Rps6ka5, Trib3,* ***Clec5a, Lgals3***	*Cd55, Dusp8, Irak2, Irs2, Nr4a1, Rnf135, Wasf1,* ***Fgf20, Hmgb1, Ifna5, Mapk10, Trim32***
Adaptive immune system	*Cblb, Dlc1, Irs2, Nr4a1, PVR, Spsb1, Trib3, Tuba4a, Tubb3*	*Dlc1, Fbxo27, Irs2, Nr4a1, Pag1, PVR, Rel, Rnf19b, Spsb1, Tuba4a, Tubb3,* ***Fgf20, Trim32, Ube2u***
Fatty acid, triacylglycerol, and ketone body metabolism	*Alas1, Dgat1, Hmgcr, Pex11a, Ptplad2, Trib3*	*Agpat9, Alas1, Ctgf, Dgat1, Fasn, Helz2, Hmgcr, Hmgcs1, Lpin1, Ppard,* ***Angptl4, Hmgcll1, Me1, Nr1d1***
Cytokine signaling in immune system	*Birc2, Cish, Dusp8, Dusp10, Gab2, Irak2, Irf7, Irs2,*	*Dusp8, Ifit1, Irak2, Irs2, Oasl, Pom121,* ***Fgf20, Ifna5***
Ppara activates gene expression	*Alas1, Hmgcr, Pex11a, Trib3*	*Alas1, Ctgf, Helz2, Hmgcr, Hmgcs1,* ***Angptl4, Me1, Nr1d1***
Mapk family signaling	*Dusp8, Dusp10, Irs2, Jun,*	*Dusp8, Irs2,* ***Cdc42ep2,***

cascades	*Myc*	***Cdc42ep3, Fgf20***
Nuclear receptor transcription pathway	*Nr4a1, Nr4a2, Nr4a3*	*Nr4a1, Nr4a2, Nr4a3, Nr5a1, Ppard,* ***Nr0b1, Nr0b2, Nr1d1***
Regulation of cholesterol biosynthesis by SREBP	*Hmgcr, Insig1*	*Fasn, Helz2, Hmgcr, Hmgcs1, Idi1, Insig1*
Phospholipid metabolism	*Dgat1, Etnk1*	*Agpat9, Dgat1, Etnk1, Lpin1*
Cholesterol biosynthesis	*Hmgcr*	*Hmgcr, Hmgcs1, Idi1*
Lipid digestion, mobilization, and transport	*Ldlr,* ***Fabp6***	*Ldlr*

Table 2. Transcription factors that were downregulated by an in utero exposure to 0.5 and 1 mg DEHP/kg BW/day at post-natal day 60. Downregulated genes are in bold.

Dose	Gene symbols
0.5 mg DEHP/kg BW/day	*Btg2, Creb3l1, Crem, Giot, Hes1, Irf7, Jun, Junb, Maf, Myc, Nfil3, Nr4a1, Nr4a2, Nr4a3, Per1, Loc680200, Trib3*
1 mg DEHP/kg BW/day	*Crebl1, Crem, Giot, Maf, Nfil3, Nr4a1, Nr4a2, Nr4a3, Nr5a1, Per1, Ppard,* ***Ddit3, Dbp, Nr0b1, Nr0b2, Nr1d1, Pou4f1, Zfp51***

Summary and Conclusions

To date, the majority of the studies on DEHP exposure in animal models have been performed utilizing doses of DEHP that may not be reflective of the amounts present in the environment. Although these studies have been crucial in identifying the anti-androgenic effects of DEHP and phthalates as a whole [103], it may be difficult to approximate human risk. Given the permanent and long-term effects of DEHP [127] as well as its ability to synergize with other phthalates in its anti-androgenic action [154], it is of importance to explore the effects of environmentally-relevant levels of DEHP. Although some groups have begun to examine exposures at lower doses [138, 155], the focus is not on early fetal exposure. This is of critical importance as the fetus is vulnerable to foreign exposures such as DEHP during development and it is hypothesized that these exposures can have long-term consequences in adult health [113].

The present data provides evidence that *in utero* exposure to 1 mg DEHP/kg BW/day, an environmentally-relevant dose, targets the fetal zona glomerulosa. Global gene array analysis of the PND60 zona glomerulosa revealed a number of deregulated genes that were associated with pathways deregulated at high doses of DEHP, suggesting the 1 mg dose, although not enough to alter circulating aldosterone levels, caused a first hit in the adrenal gland. In addition, many of these genes were found to be in common with gene changes in zona glomerulosa following potassium or ATII stimulation, further supporting the hypothesis that the aldosterone pathway is a key target of *in utero* DEHP exposure. Treatment with the secondary stressor, T0070907, resulted in a significant decrease of serum aldosterone in adult males exposed *in utero* to 1 mg DEHP/kg BW/day. Gene expression analysis of adrenals in these males revealed a decrease in

Kcnk5, *Rxrα*, and *Rxrβ* mRNA levels, suggesting they may be key genes related to the decrease in aldosterone.

The endocrine disrupting effect of DEHP on the adrenal glands is a complex mechanism that alters several adrenal pathways and has yet to be fully elucidated. However, the low dose exposure reveals a number of key genes that are affected and may be important targets that ultimately lead to adrenal dysfunction at higher doses.

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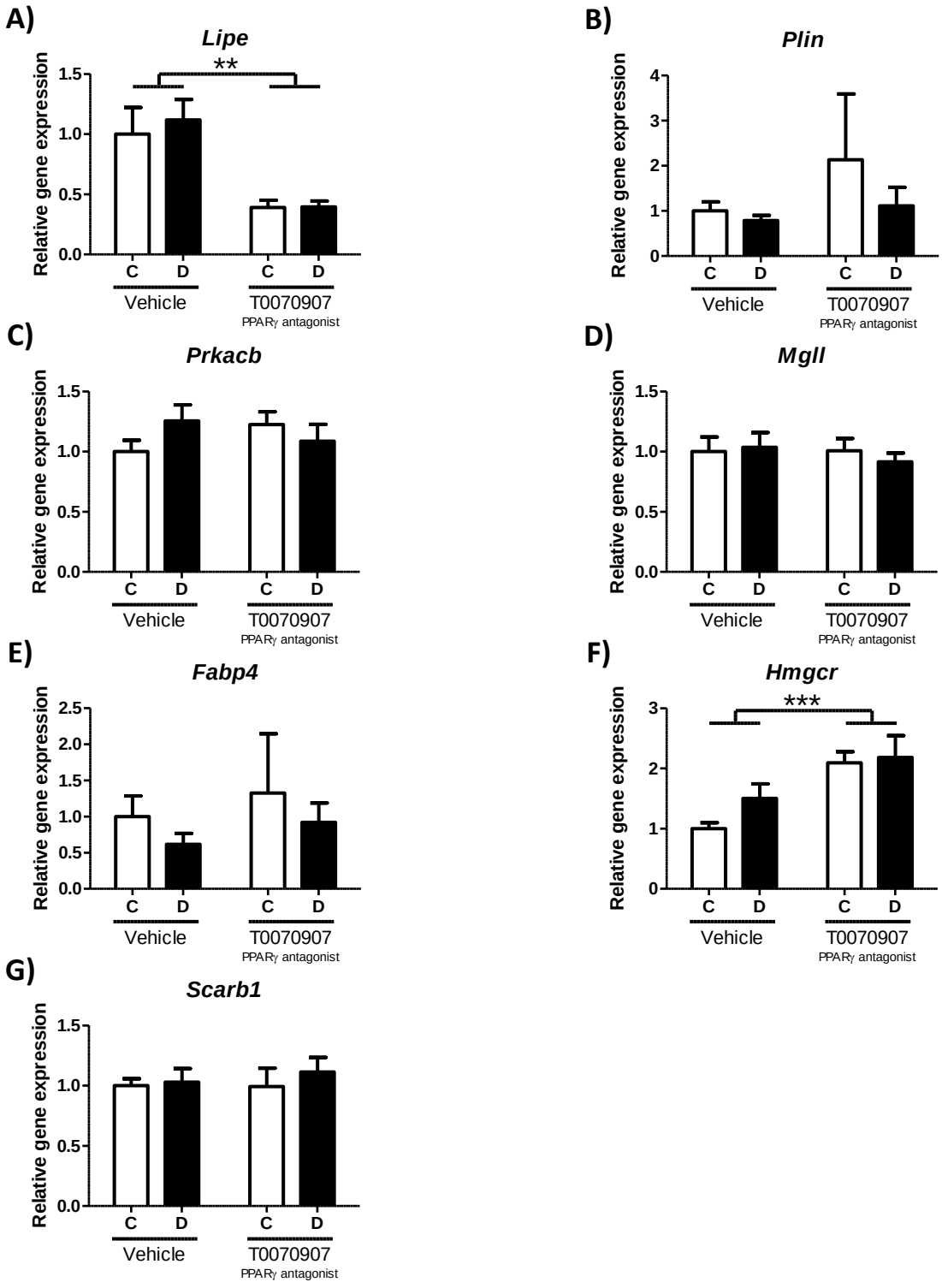
Supplemental Figures 1 and 2

Supplemental Figure 1. Relative gene expression of Plin, Prkacb, Lipe, Mgll, Fabp4, Hmgcr, and Scarb1

Gene expression shown for (A) *Plin*, (B) *Prkacb*, (C) *Lipe*, (D) *Mgll*, (E) *Fabp4*, (F) *Hmgcr*, and (G) *Scarb1*. Data represent means ± SEM; $n \geq 5$; bars indicate significance by Student's T-test or 2-way ANOVA, $^{**}p < 0.01$; $^{***}p < 0.001$.

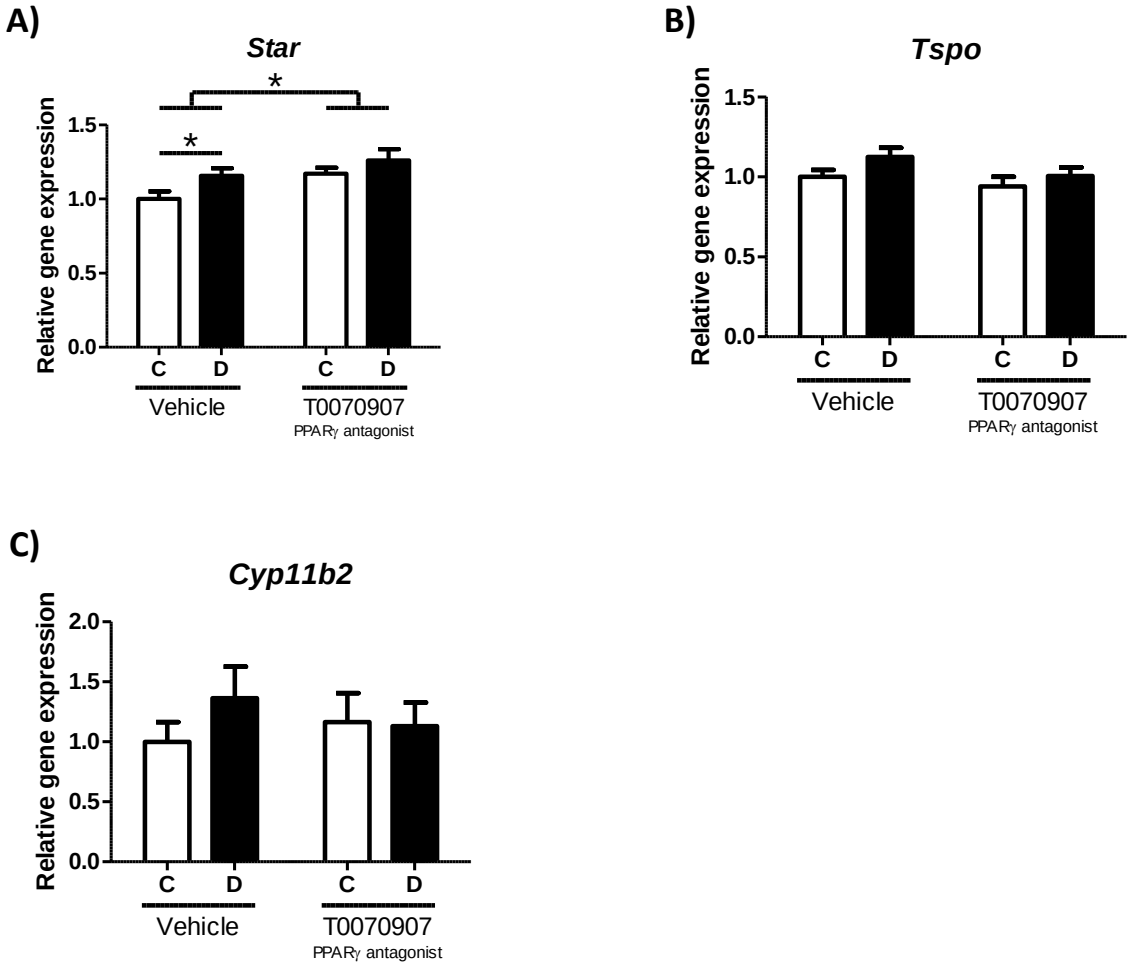
Supplemental Figure 1. Relative gene expression levels shown for Plin, Prkacb, Lipe, Mgll, Fabp4, Hmgcr, and Scarb1



Supplemental Figure 2. Relative gene expression of Star, Tspo, Cyp11b2

Gene expression shown for (A) *Star,* (B) *Tspo,* and (C) *Cyp11b2.* Data represent means ± SEM; $n \geq 5$; bars indicate significance by Student's T-test or 2-way ANOVA, $*p < 0.05$.

Supplemental Figure 2. Relative gene expression levels shown for Star, Tspo, Cyp11b2



Supplemental Table 1

Supplemental Table 1. Taqman quantitative PCR probes used for gene expression analysis.

Gene symbol	RefSeq	TaqMan Reference
Agtr1a	NM_030985.4	Rn01435427_m1
Agtr1b	NM_031009.2	Rn02132799_s1
Agtr2	NM_012494.3	Rn00560677_s1
Cyp11b2	NM_012538.2	Rn02396730_g1
Fabp4	NM_053365.1	Rn04219585_m1
Gapdh	NM_017008.3	4352338E
Hmgcr	NM_013134.2	Rn00565598_m1
Insig1	NM_022392.1	Rn00574380_m1
Kcnk5	NM_001039516.2	Rn01755927_m1
Kcnn2	NM_019314.1	Rn00570910_m1
Ldlr	NM_175762.2	Rn00598442_m1
Lipe	NM_012859.1	Rn00689222_m1
Mgll	NM_138502.2	Rn00593297_m1
Nr4a1	NM_024388.2	Rn01533237_m1
Nr4a3	NM_031628.1	Rn00569312_g1
Plin	NM_013094.1	Rn00558672_m1
Pparα	NM_013196.1	Rn00566193_m1
Pparβ	NM_013141.2 R	Rn00565707_m1
Pparγ	NM_013124.3	Rn00440945_m1
Prkacb	NM_001077645.1	Rn01748544_m1

Rxrα	NM_012805.2	Rn00441185_m1
Rxrβ	NM_206849.3	Rn01399560_m1
Rxrγ	NM_031765.1	Rn01483465_m1
Scarb1	NM_031541.1	Rn00580588_m1
Star	NM_031558.3	Rn00580695_m1
Tspo	NM_012515.2	Rn00560892_m1