Mechanism of action of phthalate endocrine disruptors in male reproductive development

A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

by

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Declaration

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* Ravinder Anand-Ivell, Kee Heng, Bettina Hafen, Brian Setchell, and Richard Ivell. Dynamics of INSL3 peptide expression in the rodent testis. *Biology of Reproduction*, 2009. **81**(3): p. 480-7.

Kee Heng

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Abstract

Phthalates are plasticizers, commonly found in cosmetics, food-wrapping, personal care products and medical devices, and have recently been associated with reduced anogenital distance in male infants, reduced testosterone and altered behavior in boys. In rodents, *in utero* phthalate exposure disrupts the development of the internal and external male reproductive phenotype in the progeny. Testicular Leydig cells may be the primary target of phthalate action, since they often exhibit abnormal aggregation and reduced insulin-like factor 3 (INSL3). Moreover, microarray studies suggest that they are amongst the earliest testicular cell types affected by phthalate treatment, though acute effects on mature cells appear to be minor. The overall aim of this project was to assess the effect of phthalate, with the synthetic non-steroidal estrogen, diethylstilbestrol (DES) as control, on Leydig cell differentiation.

First a time-resolved fluorescent immunoassay (TRFIA) for rodent INSL3, a specific marker of Leydig cell differentiation, was developed, which was highly specific for rat and mouse INSL3 in body fluids or in cell culture medium. Besides showing that INSL3 is a reliable marker for aging Leydig cells, it accurately reflected the differentiation of Leydig cells during puberty.

The second aim of this project assessed whether the kinetics of new Leydig cell differentiation following their ablation by ethane dimethane sulfonate (EDS) was affected by dibutyl phthalate (DBP) treatment at an early stage of Leydig cell regeneration. mRNA expression of Leydig cell markers such as LH receptor (LHR), cytochrome P450 17-alpha-hydroxylase/17,20 lyase (Cyp17a1) and 11-beta-hydroxysteroid dehydrogenase type 1 (Hsd11b1) in the DBP-treated animals were increased, likely due to an increase in cell

proliferation since Leydig cells in treated animals exhibited more clustering and higher numerical density compared to controls.

The long-term effect of DBP on the adult Leydig cell population following *in utero* and lactational exposure was also investigated since most studies have concentrated on acute early effects of phthalates. Maternal DBP treatment appeared to accelerate Leydig cell development, especially when Leydig cells are actively proliferating or differentiating, largely due to an increased proliferation of Leydig cells. The consequences of such treatment do not persist to adulthood, since circulating INSL3 as well as mRNA expression of various Leydig cell markers were comparable in all treatment groups at postnatal day 90 (PND90).

Finally, a long term *in vitro* model of Leydig cell differentiation was established, using undifferentiated adult-type Leydig cells isolated from day 10 rats. Preliminary experiments with DBP and its main metabolite, monobutyl phthalate (MBP), showed that both compounds were probably inhibitory to Leydig cell differentiation in culture in the presence of human chorionic gonadotropin (hCG). The precise mechanism by which these compounds slowed Leydig cell differentiation will be determined in future studies.

Collectively, the findings of this thesis strongly imply that differentiating/developing adult-type Leydig cells are indeed direct targets of endocrine disruption. This thesis has also demonstrated that the EDS model and an *in vitro* Leydig cell differentiation model will be very useful in delineating the underlying mechanism of phthalate action.

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Publications arising from this Thesis

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Ravinder Anand-Ivell, Kee Heng, Bettina Hafen, Brian Setchell, and Richard Ivell. Dynamics of INSL3 peptide expression in the rodent testis. *Biology of Reproduction*, 2009. **81**(3): p. 480-7.

Article submitted to scientific journal

Kee Heng, Ravinder Anand-Ivell and Richard Ivell. The endocrine disruptors dibutyl phthalate (DBP) and diethylstilbestrol (DES) influence Leydig cell regeneration following ethane dimethane sulfonate (EDS) treatment of adult male rats. *International Journal of Andrology* (submitted June 2011).

Abstracts published in the proceedings of scientific meetings

Kee Heng, Bettina Hafen, Richard Ivell and Ravinder Anand-Ivell. Development of a model system to assess the regulation of Leydig stem cell differentiation. Gordon Research Conference: Environmental Endocrine Disruptors, Les Diablerets, Switzerland, May 2010.

Ravinder Anand-Ivell, Kee Heng, Bettina Hafen, Brian Setchell and Richard Ivell. Secreted insulin-like peptide 3 (INSL3) and its role in postnatal development. 16th European Workshop on Molecular and Cellular Endocrinology of the Testis, Island of Elba, Italy, May 2010.

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differentiation. 40th annual conference of the Society for Reproductive Biology (SRB), Adelaide, Australia, August 2009.

Ravinder Anand-Ivell, Kee Heng, Bettina Hafen, Brian Setchell and Richard Ivell. Postnatal development and dynamics of the secreted Leydig cell peptide hormone insulin-like peptide 3 (INSL3) in rodents. 40th annual conference of the Society for Reproductive Biology (SRB), Adelaide, Australia, August 2009.

Abstract arising not directly from the work in this thesis

Richard Ivell, Damien Hunter, Kee Heng, Navdeep Mann, and Ravinder Anand-Ivell. Maternal exposure to phthalate and/or diethylstilbestrol leads to long-term changes in hypothalamic gene expression and adult behavior in male and female offspring. 44th annual meeting of the Society for the Study of Reproduction (SSR), Oregon, USA, accepted for poster presentation in July 2011.

Abbreviations

3αHSD	3-alpha-hydroxysteroid dehydrogenase
ACTH	Adrenocorticotropic hormone
AGD	Anogenital distance
AMH	Anti-Mullerian hormone
ALCs	Adult Leydig cells
AR	Androgen receptor
BBP	Benzyl butyl phthalate
BSA	Bovine serum albumin
b.w.	Body weight
cDNA	Complementary DNA
Chrna4	Nicotinic acetylcholine receptor alpha 4
CIS	Carcinoma in situ
Cyp11a1	Cytochrome P450 cholesterol side chain cleavage enzyme
Cyp17a1	Cytochrome P450 17-alpha-hydroxylase/17,20 lyase
CV	Coefficient of variation
Dax1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DBP	Dibutyl phthalate
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DES	Diethylstilbestrol
DHH	Desert hedgehog
DHT	Dihydrotestosterone
DiBP	Diisobutyl phthalate
DINP	Diisononyl phthalate
DMEM	Dulbecco's Modified Eagle Medium
DMP	Dimethyl phthalate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease

dNTP	Deoxyribonucleotide
DOTP	Dioctyl terephthalate
DPP	Dipentyl phthalate
E	Embryonic day
EDS	Ethane dimethane sulfonate
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FLCs	Fetal Leydig cells
GATA4	GATA binding protein 4
GD	Gestation day
GnRH	Gonadotropin-releasing hormone
GTC	Guanidine Thiocyanate
H&E	Haematoxylin and eosin
hCG	Human chorionic gonadotropin
hpg	Hypogonadal
HPG	Hypothalamic-pituitary-gonadal
HPLC	High-performance liquid chromatography
HRP	Horse radish peroxidase
Hsd3b1	3-beta-hydroxysteroid dehydrogenase
Hsd11b1	11-beta-hydroxysteroid dehydrogenase type 1
Hsd17b3	17-beta-hydroxysteroid dehydrogenase 3
Hsd17b10	17-beta-hydroxysteroid dehydrogenase type 10
IGF-1	Insulin-like growth factor-1
IGF-2	Insulin-like growth factor-2
IgG	Immunoglobulin G
ILCs	Immature Leydig cells
INSL3	Insulin-like factor 3
i.p.	Intraperitoneal
JAK3	Janus Kinase 3
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
MBP	Monobutyl phthalate
MC_2R	Melanocortin type 2 receptor
MEHP	Mono(2-ethylhexyl) phthalate

MNG	Multinucleated gonocyte
MQ	MilliQ
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NA	Numerical aperture
NE	Normalized expression
Nr4a1	Nuclear receptor subfamily 4 group A member 1
OD	Optical density
PAS	Periodic Acid Schiff's
PBR	Peripheral-type benzodiazepine receptor
PBS	Phosphate buffered saline
PDGFA	Platelet-derived growth factor A
PDGF-BB	Platelet derived growth factor BB
PDGFRα	Platelet-derived growth factor receptor α
PGCs	Primordial germ cells
PLCs	Progenitor Leydig cells
PND	Postnatal day
PPARα	Peroxisome proliferator-activated receptor α
PVC	Polyvinyl chloride
RIA	Radioimmunoassay
RNA	Ribonucleic acid
Rps27a	Ribosomal protein S27a
RT-PCR	Reverse transcription polymerase chain reaction
RXFP2	Relaxin/insulin-like family peptide receptor 2
s.c.	Subcutaneous
SCO	Sertoli-cell only
SD	Sprague Dawley
SER	Smooth endoplasmic reticulum
Sf1	Steroidogenic factor 1
SLCs	Stem Leydig cells
SOX9	Sex-determining region Y-box containing gene 9
SRY	Sex-determining region Y
StAR	Steroidogenic acute regulatory protein
TDS	Testicular dysgenesis syndrome

Tfm	Testicular feminization
TMB	3,3’,5,5’-tetramethylbenzidine
TRFIA	Time-resolved fluorescent immunoassay
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization
Wt1	Wilms tumour 1

Units

°C	Degree Celsius
µl	Microlitre
µg	Microgram
µm	Micron / Micrometre
µsec	Microsecond
bp	Base pairs
cm	Centimetre
cps	Counts per second
g	Gram
$\times g$	Relative centrifugal force
G	Gauge
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
L	Litre
M	Molar
mg	Milligram
min	Minute
mIU	Milli International Unit
ml	Millilitre
mm	Millimetre
mM	Millimolar
n	Number
ng	Nanogram
nm	Nanometer

nM	Nanomolar
pg	Picogram
rpm	Revolutions per minute
sec	Second
U	Unit