AOP ID and Title:

SNAPSHOT

Created at: 2019-01-04 08:55

AOP 51: PPARα activation leading to impaired fertility in adult male rodents

Short Title: PPAR and reproductive toxicity

Authors

Malgorzata Nepelska, Sharon Munn, Brigitte Landesmann Systems Toxicology Unit, Institute for Health and Consumer Protection, Joint Research Centre, European Commission, Ispra, Varese, Italy

Status

Author status	OECD status	OECD project	SAAOP status
Under development: Not open for comment. Do not cite	Under Development	1.21	Included in OECD Work Plan

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	227	Activation, PPARα (https://aopwiki.org/events/227)	Activation, PPARα
2	KE	414	Increase, Luteinizing hormone (LH) (https://aopwiki.org/events/414)	Increase, Luteinizing hormone (LH)
3	KE	415	Hyperplasia, Leydig cell (https://aopwiki.org/events/415)	Hyperplasia, Leydig cell
4	KE	416	Increase proliferation, Leydig cell (https://aopwiki.org/events/416)	Increase proliferation, Leydig cell
5	KE	446	Reduction, testosterone level (https://aopwiki.org/events/446)	Reduction, testosterone level
6	KE	413	Reduction, Testosterone synthesis in Leydig cells (https://aopwiki.org/events/413)	Reduction, Testosterone synthesis in Leydig cells
7	KE	447	Reduction, Cholesterol transport in mitochondria (https://aopwiki.org/events/447)	Reduction, Cholesterol transport in mitochondria
8	AO	406	impaired, Fertility (https://aopwiki.org/events/406)	impaired, Fertility

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increase proliferation, Leydig cell (https://aopwiki.org/relationships/398)	adjacent	Hyperplasia, Leydig cell	Moderate	
Reduction, Testosterone synthesis in Leydig cells (https://aopwiki.org/relationships/439)	adjacent	Reduction, testosterone level	High	
Reduction, Cholesterol transport in mitochondria (https://aopwiki.org/relationships/438)	adjacent	Reduction, Testosterone synthesis in Leydig cells	Moderate	
Reduction, testosterone level (https://aopwiki.org/relationships/460)	non-adjacent	Increase, Luteinizing hormone (LH)	Moderate	
Increase, Luteinizing hormone (LH) (https://aopwiki.org/relationships/461)	non-adjacent	Increase proliferation, Leydig cell	Moderate	
Hyperplasia, Leydig cell (https://aopwiki.org/relationships/462)	non-adjacent	impaired, Fertility	Moderate	

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate
Juvenile	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

Sex Applicability

Sex	Evidence
Male	High

References

Appendix 1

List of MIEs in this AOP

Event: 227: Activation, PPARα (https://aopwiki.org/events/227)

Short Name: Activation, PPARα

Key Event Component

Process	Object	Action
peroxisome proliferator activated receptor signaling pathway	peroxisome proliferator-activated receptor alpha	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:18 - PPARα activation in utero leading to impaired fertility in males (https://aopwiki.org/aops/18)	MolecularInitiatingEvent
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	MolecularInitiatingEvent
Aop:61 - NFE2L2/FXR activation leading to hepatic steatosis (https://aopwiki.org/aops/61)	KeyEvent
Aop:37 - PPARalpha-dependent liver cancer (https://aopwiki.org/aops/37)	MolecularInitiatingEvent

Stressors

Name
Di(2-ethylhexyl) phthalate
Mono(2-ethylhexyl) phthalate

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Fibrates are ligands of PPARα (Staels et al. 1998).

Phthalates

MHEP (CAS 4376-20-9) directly binds *in vitro* to PPARα (Lapinskas et al. 2005) and activates this receptor in transactivation assays PPARα (Lapinskas et al. 2005), (Maloney and Waxman 1999), (Hurst and Waxman 2003), (Bility et al. 2004), (Lampen, Zimnik, and Nau 2003), (Venkata et al. 2006)]. DEHP (CAS 117-81-7) has not been found to bind and activate PPARα (Lapinskas et al. 2005), (Maloney and Waxman 1999). However, the recent studies shown activation of PPARα (ToxCastTM Data).

Notably, PPARα are responsive to DEHP *in vitro* as they are translocated to the nucleus (in primary Sertoli cells) (Dufour et al. 2003), (Bhattacharya et al. 2005). Expression of PPARα [mRNA and protein] has been reported to be also modulated by phthtalates: (to be up-regulated *in vivo* upon DEHP treatment (Xu et al. 2010) and down-regulated by Diisobutyl phthalate (DiBP) (Boberg et al. 2008)).

Perfluorooctanoic Acid (PFOA) is known to activate PPARα (Vanden Heuvel et al. 2006).

Organotin

Tributyltin (TBT) activates all three heterodimers of PPAR with RXR, primarily through its interaction with RXR (le Maire et al. 2009)

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

PPARα has been identified in frog (Xenopus laevis), mouse, human, rat, fish, hamster and chicken (reviewed in (Wahli and Desvergne 1999)).

Key Event Description

Biological state

The Peroxisome Proliferator Activated receptor α (PPARα) belongs to the Peroxisome Proliferator Activated receptors (PPARs; NR1C) (https://aopwiki.org/wiki/index.php/Peroxisome_Proliferator_Activated_receptors_(PPARs;_NR1C)) steroid/thyroid/retinoid receptor superfamily of transcription factors.

Biological compartments

PPARα is expressed in high levels in tissues that perform significant catabolism of fatty acids (FAs), such as brown adipose tissue, liver, heart, kidney, and intestine (Michalik et al. 2006). The receptor is present also in skeletal muscle, intestine, pancreas, lung, placenta and testes (Mukherjee et al. 1997), (Schultz et al. 1999).

General role in biology

PPARs are activated by fatty acids and their derivatives; they are sensors of dietary lipids and are involved in lipid and carbohydrate metabolism, immune response and peroxisome proliferation (Wahli and Desvergne 1999), (Evans, Barish, & Wang, 2004). PAPRα is a also a target of hypothalamic hormone signalling and was found to play a role in embryonic development (Yessoufou and Wahli 2010).

Fibrates, activators of PPARα, are commonly used to treat hypertriglyceridemia and other dyslipidemic states as they have been shown to decrease circulating lipid levels (Lefebvre et al. 2006).

How it is Measured or Detected

Binding of ligands to PPARα is measured using binding assays in vitro and in silico, whereas the information about functional activation is derived from transactivation assays (e.g. transactivation assay with reporter gene) that demonstrate functional activation of a nuclear receptor by a specific compound. Binding of agonists within the ligand-binding site of PPARs causes a conformational change of nuclear receptor that promotes binding to transcriptional co-activators. Conversely, binding of antagonists results in a conformation that favours the binding of co-repressors (Yu and Reddy 2007), (Viswakarma et al. 2010). Transactivation assays are performed using transient or stably transfected cells with the PPARα expression plasmid and a reporter plasmid, respectively. There are also other methods that have been used to measure PPARα activity, such as the Electrophoretic Mobility Shift Assay (EMSA) or commercially available PPARα transcription factor assay kits, see Table 1. The transactivation (stable transfection) assay provides the most applicable OECD Level 2 assay (i.e. In vitro assays providing mechanistic data) aimed at identifying the initiating event leading to an adverse outcome (LeBlanc, Norris, and Kloas 2011). Currently no internationally validated assays for regulatory purposes are available.

Key event	**PPARα activation**				
What is measured?	Ligand Binding		Transcriptional activity		
Method/test category	molecular modelling	binding assay	transactivation reporter gene assay	transcription factor assay	
Method/test name	molecular modelling; docking	Scintillation proximity binding assay	luciferase reporter gene assay	PPARα (mouse/rat) Reporter Assay Kit	Electrophoretic Mobility Shift Assay (EMSA)

Test environment	*In silico*	*In vitro*	*In vitro*			*In vitro, ex vivo*	
Test principle	Computational simulation of a candidate ligand binding to a receptor, Predicts the strength of association or binding affinity.	Direct binding indicating the mode of action for PPARα	Quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in PPAR functional activity.			PPARα once activated by a ligand, the receptor binds to a promoter element in the gene for target gene and activates its transcription. The DNA-bound (activated) PPAR is measured.	
Test outcome	A binding interaction between a small molecule ligand and an enzyme protein may result in activation or inhibition of the enzyme. If the protein is a receptor, ligand binding may result in agonism or antagonism of the normal activity of the receptor.	Assesses the ability of compounds to bind to PPARα. Identifies the modulators of PPARα.	The changes in activity of reporter gene levels functionally linked to a PPAR-responsive element/promoter gives information about the nature of the PPAR activation.			Protein: DNA binding, DNA binding activity	
Test background	Predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. Knowledge of the preferred orientation in turn may be used to predict the strength of association or binding affinity between two molecules using, for example, scoring functions.	This assay determines whether compounds interact directly with PPARs. The type of beads that are involved in the SPA are microscopic in size and within the beads, there is a scintillant which emits light when it is stimulated. Stimulation occurs when radio-labelled molecules interact and bind to the surface of the bead and trigger the bead to emit light.	PPARα/γ COS-1cell transactivation assay (transient transfection with human or mouse PPARα/γ expression plasmid and pHD(x3)-Luc reporter plasmid	(PPRE)3-luciferase reporter construct C2C12	Proprietary rodent cell line expressing the mouse/rat PPARα	Transcriptional activity of PPARα can be assessed using commercially available kits like e.g. PPAR-α transcription factor assay kit.	Gene regulation and determining protein: DNA interactions are detected by the EMSA. EMSA can be used qualitatively to identify sequence-specific DNA-binding proteins (such as transcription factors) in crude lysates and, in conjunction with mutagenesis, to identify the important binding sequences within a given gene upstream regulatory region. EMSA can also be utilized quantitatively to measure thermodynamic and kinetic parameters.
Assay type	Quantitative	Qualitative	Quantitative	Quantitative	Quantitative	Quantitative	Quantitative

Application domain	Virtual screening	*In vitro* screening	*In vitro* Screening, functional studies activity (reported use: agonist)		*In vitro* Screening functional activity (antagonist/agonist)		
Ref	(Feige et al. 2007), (Kaya et al. 2006)	(Lapinskas et al. 2005), (Wu, Gao, and Wang 2005)	(Maloney and Waxman 1999)	(Feige et al. 2007)	Indigobiosciences (http://indigobiosciences.com/products/ppar-products/mouse-ppar-alpha-mpparα-nr1c1/)	Abcam (http://www.abcam.com/ppar-alpha-transcription-factor-assay-kit-ab133107.html)	

Table 1 Summary of the chosen methods to measure the PPARα activation.

References

Bhattacharya, Nandini, Jannette M Dufour, My-Nuong Vo, Janice Okita, Richard Okita, and Kwan Hee Kim. 2005. “Differential Effects of Phthalates on the Testis and the Liver.” Biology of Reproduction 72 (3) (March): 745–54. doi:10.1095/biolreprod.104.031583.

Bility, Moses T, Jerry T Thompson, Richard H McKee, Raymond M David, John H Butala, John P Vanden Heuvel, and Jeffrey M Peters. 2004. “Activation of Mouse and Human Peroxisome Proliferator-Activated Receptors (PPARs) by Phthalate Monoesters.” Toxicological Sciences : An Official Journal of the Society of Toxicology 82 (1) (November): 170–82. doi:10.1093/toxsci/kfh253.

Dufour, Jannette M, My-Nuong Vo, Nandini Bhattacharya, Janice Okita, Richard Okita, and Kwan Hee Kim. 2003. “Peroxisome Proliferators Disrupt Retinoic Acid Receptor Alpha Signaling in the Testis.” Biology of Reproduction 68 (4) (April): 1215–24. doi:10.1095/biolreprod.102.010488.

Feige, Jérôme N, Laurent Gelman, Daniel Rossi, Vincent Zoete, Raphaël Métivier, Cicerone Tudor, Silvia I Anghel, et al. 2007. “The Endocrine Disruptor Monoethyl-Hexyl-Phthalate Is a Selective Peroxisome Proliferator-Activated Receptor Gamma Modulator That Promotes Adipogenesis.” The Journal of Biological Chemistry 282 (26) (June 29): 19152–66. doi:10.1074/jbc.M702724200.

Hurst, Christopher H, and David J Waxman. 2003. “Activation of PPARalpha and PPARgamma by Environmental Phthalate Monoesters.” Toxicological Sciences : An Official Journal of the Society of Toxicology 74 (2) (August): 297–308. doi:10.1093/toxsci/kfg145.

Kaya, Taner, Scott C Mohr, David J Waxman, and Sandor Vajda. 2006. “Computational Screening of Phthalate Monoesters for Binding to PPARgamma.” Chemical Research in Toxicology 19 (8) (August): 999–1009. doi:10.1021/tx050301s.

Lampen, Alfonso, Susan Zimnik, and Heinz Nau. 2003. “Teratogenic Phthalate Esters and Metabolites Activate the Nuclear Receptors PPARs and Induce Differentiation of F9 Cells.” Toxicology and Applied Pharmacology 188 (1) (April): 14–23. doi:10.1016/S0041-008X(03)00014-0.

Lapinskas, Paula J., Sherri Brown, Lisa M. Leesnitzer, Steven Blanchard, Cyndi Swanson, Russell C. Cattley, and J. Christopher Corton. 2005. “Role of PPARα in Mediating the Effects of Phthalates and Metabolites in the Liver.” Toxicology 207 (1): 149–163.

Le Maire, Albane, Marina Grimaldi, Dominique Roecklin, Sonia Dagnino, Valérie Vivat-Hannah, Patrick Balaguer, and William Bourguet. 2009. “Activation of RXR-PPAR Heterodimers by Organotin Environmental Endocrine Disruptors.” EMBO Reports 10 (4) (April): 367–73. doi:10.1038/embor.2009.8.

LeBlanc, GA, DO Norris, and W Kloas. 2011. “Detailed Review Paper State of the Science on Novel In Vitro and In Vivo Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors” (178).

Lefebvre, Philippe, Giulia Chinetti, Jean-Charles Fruchart, and Bart Staels. 2006. “Sorting out the Roles of PPAR Alpha in Energy Metabolism and Vascular Homeostasis.” The Journal of Clinical Investigation 116 (3) (March): 571–80. doi:10.1172/JCI27989.

Maloney, Erin K., and David J. Waxman. 1999. “Trans-Activation of PPARα and PPARγ by Structurally Diverse Environmental Chemicals.” Toxicology and Applied Pharmacology 161 (2): 209–218.

Michalik, Liliane, Johan Auwerx, Joel P Berger, V Krishna Chatterjee, Christopher K Glass, Frank J Gonzalez, Paul A Grimaldi, et al. 2006. “International Union of Pharmacology. LXI. Peroxisome Proliferator-Activated Receptors.” Pharmacological Reviews 58 (4) (December): 726–41. doi:10.1124/pr.58.4.5.

Mukherjee, R, L Jow, G E Croston, and J R Paterniti. 1997. “Identification, Characterization, and Tissue Distribution of Human Peroxisome Proliferator-Activated Receptor (PPAR) Isoforms PPARgamma2 versus PPARgamma1 and Activation with Retinoid X Receptor Agonists and Antagonists.” The Journal of Biological Chemistry 272 (12) (March 21): 8071–6.

Schultz, R, W Yan, J Toppari, A Völkl, J A Gustafsson, and M Pelto-Huikko. 1999. “Expression of Peroxisome Proliferator-Activated Receptor Alpha Messenger Ribonucleic Acid and Protein in Human and Rat Testis.” Endocrinology 140 (7) (July): 2968–75. doi:10.1210/endo.140.7.6858.

Staels, B., J. Dallongeville, J. Auwerx, K. Schoonjans, E. Leitersdorf, and J.-C. Fruchart. 1998. “Mechanism of Action of Fibrates on Lipid and Lipoprotein Metabolism.” Circulation 98 (19) (November 10): 2088–2093. doi:10.1161/01.CIR.98.19.2088.

ToxCastTM Data. “ToxCastTM Data.” US Environmental Protection Agency. http://www.epa.gov/ncct/toxcast/data.html (http://www.epa.gov/ncct/toxcast/data.html)

Vanden Heuvel, John P, Jerry T Thompson, Steven R Frame, and Peter J Gillies. 2006. “Differential Activation of Nuclear Receptors by Perfluorinated Fatty Acid Analogs and Natural Fatty Acids: A Comparison of Human, Mouse, and Rat Peroxisome Proliferator-Activated Receptor-Alpha, -Beta, and -Gamma, Liver X Receptor-Beta, and Retinoid X Rec.” Toxicological Sciences : An Official Journal of the Society of Toxicology 92 (2) (August): 476–89. doi:10.1093/toxsci/kfl014.

Venkata, Nagaraj Gopisetty, Jodie a Robinson, Peter J Cabot, Barbara Davis, Greg R Monteith, and Sarah J Roberts-Thomson. 2006. “Mono(2-Ethylhexyl)phthalate and Mono-N-Butyl Phthalate Activation of Peroxisome Proliferator Activated-Receptors Alpha and Gamma in Breast.” Toxicology Letters 163 (3) (June 1): 224–34. doi:10.1016/j.toxlet.2005.11.001.

Viswakarma, Navin, Yuzhi Jia, Liang Bai, Aurore Vluggens, Jayme Borensztajn, Jianming Xu, and Janardan K Reddy. 2010. “Coactivators in PPAR-Regulated Gene Expression.” PPAR Research 2010 (January). doi:10.1155/2010/250126.

Wahli, Walter, and B Desvergne. 1999. “Peroxisome Proliferator-Activated Receptors: Nuclear Control of Metabolism.” Endocrine Reviews 20 (5) (October): 649–88. Wu, Bin, Jie Gao, and Ming-wei Wang. 2005. “Development of a Complex Scintillation Proximity Assay for High-Throughput Screening of PPARgamma Modulators.” Acta Pharmacologica Sinica 26 (3) (March): 339–44. doi:10.1111/j.1745-7254.2005.00040.x.

Xu, Chuan, Ji-An Chen, Zhiqun Qiu, Qing Zhao, Jiaohua Luo, Lan Yang, Hui Zeng, et al. 2010. “Ovotoxicity and PPAR-Mediated Aromatase Downregulation in Female Sprague-Dawley Rats Following Combined Oral Exposure to Benzo[a]pyrene and Di-(2-Ethylhexyl) Phthalate.” Toxicology Letters 199 (3) (December 15): 323–32. doi:10.1016/j.toxlet.2010.09.015.

Yessoufou, a, and W Wahli. 2010. “Multifaceted Roles of Peroxisome Proliferator-Activated Receptors (PPARs) at the Cellular and Whole Organism Levels.” Swiss Medical Weekly 140 (September) (January): w13071. doi:10.4414/smw.2010.13071.

Yu, Songtao, and Janardan K Reddy. 2007. “Transcription Coactivators for Peroxisome Proliferator-Activated Receptors.” Biochimica et Biophysica Acta 1771 (8) (August): 936–51. doi:10.1016/j.bbalip.2007.01.008.

List of Key Events in the AOP

Event: 414: Increase, Luteinizing hormone (LH) (https://aopwiki.org/events/414)

Short Name: Increase, Luteinizing hormone (LH)

Key Event Component

Process	Object	Action
hormone biosynthetic process	Luteinizing hormone	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
Leydig cell

Event: 415: Hyperplasia, Leydig cell (https://aopwiki.org/events/415)

Short Name: Hyperplasia, Leydig cell

Key Event Component

Process	Object	Action
hyperplasia		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
Leydig cell

Event: 416: Increase proliferation, Leydig cell (https://aopwiki.org/events/416)

Short Name: Increase proliferation, Leydig cell

Key Event Component

Process	Object	Action
cell proliferation		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
Leydig cell

Event: 446: Reduction, testosterone level (https://aopwiki.org/events/446)

Short Name: Reduction, testosterone level

Key Event Component

Process	Object	Action
	testosterone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	KeyEvent
Aop:18 - PPARα activation in utero leading to impaired fertility in males (https://aopwiki.org/aops/18)	KeyEvent
Aop:64 - Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility (https://aopwiki.org/aops/64)	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
blood

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

Key enzymes needed for testosterone production first appear in the common ancestor of amphioxus and vertebrates (Baker 2011). Consequently, this key event is applicable to most vertebrates, including humans.

Key Event Description

Biological state

Testosterone (T) is a steroid hormone from the androgen group. T serves as a substrate for two metabolic pathways that produce antagonistic sex steroids.

Biological compartments

Testosterone is synthesized by the gonads and other steroidogenic tissues (e.g., brain, adipose), acts locally and/or is transported to other tissues via blood circulation. Leydig cells are the testosterone-producing cells of the testis.

General role in biology

Androgens, the main male sex steroids, are the critical factors responsible for the development of the male phenotype during embryogenesis and for the achievement of sexual maturation at puberty. In adulthood, androgens remain essential for the maintenance of male reproductive function and behaviour. Apart from their effects on reproduction, androgens affect a wide variety of non-reproductive tissues such as skin, bone, muscle, and brain (Heemers, Verhoeven, & Swinnen, 2006). Androgens, principally T and 5α-dihydrotestosterone (DHT), exert most of their effects by interacting with a specific receptor, the androgen receptor (AR), for review see (Murashima, Kishigami, Thomson, & Yamada, 2015). On the one hand, testosterone can be reduced by 5α-reductase to produce 5α dihydrotestosterone (DHT). On the other hand, testosterone can be aromatized to generate estrogens. Testosterone effects can also be classified by the age of usual occurrence, postnatal effects in both males and females are mostly dependent on the levels and duration of circulating free testosterone.

How it is Measured or Detected

Testosterone can be measured by immunoassays and by isotope-dilution gas chromatography-mass spectrometry in serum (Taieb et al., 2003), (Paduch et al., 2014). Testosterone levels are measured i.a. in: Fish Lifecycle Toxicity Test (FLCTT) (US EPA OPPTS 850.1500), Male pubertal assay (PP Male Assay) (US EPA OPPTS 890.1500), OECD TG 441: Hershberger Bioassay in Rats (H Assay).

References

Heemers, H. V, Verhoeven, G., & Swinnen, J. V. (2006). Androgen activation of the sterol regulatory element-binding protein pathway: Current insights. Molecular Endocrinology (Baltimore, Md.), 20(10), 2265–77. doi:10.1210/me.2005-0479

Murashima, A., Kishigami, S., Thomson, A., & Yamada, G. (2015). Androgens and mammalian male reproductive tract development. Biochimica et Biophysica Acta, 1849(2), 163–170. doi:10.1016/j.bbagrm.2014.05.020

Paduch, D. A., Brannigan, R. E., Fuchs, E. F., Kim, E. D., Marmar, J. L., & Sandlow, J. I. (2014). The laboratory diagnosis of testosterone deficiency. Urology, 83(5), 980–8. doi:10.1016/j.urology.2013.12.024

Taieb, J., Mathian, B., Millot, F., Patricot, M.-C., Mathieu, E., Queyrel, N., … Boudou, P. (2003). Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography-mass spectrometry in sera from 116 men, women, and children. Clinical Chemistry, 49(8), 1381–95.

Event: 413: Reduction, Testosterone synthesis in Leydig cells (https://aopwiki.org/events/413)

Short Name: Reduction, Testosterone synthesis in Leydig cells

Key Event Component

Process	Object	Action
testosterone biosynthetic process	testosterone	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	KeyEvent
Aop:18 - PPARα activation in utero leading to impaired fertility in males (https://aopwiki.org/aops/18)	KeyEvent
Aop:64 - Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility (https://aopwiki.org/aops/64)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
testosterone secreting cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mice	Mus sp.	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10095)

Key enzymes needed for testosterone production first appear in the common ancestor of amphioxus and vertebrates (Baker 2011). Consequently, this key event is applicable to most vertebrates, including humans.

Key Event Description

Biological state

Testosterone is a steroid hormone from the androgen group and is found in humans and other vertebrates.

Biological compartments

In humans and other mammals, testosterone is secreted primarily by the testicles of males and, to a lesser extent, the ovaries of females and other steroidogenic tissues (e.g., brain, adipose). It either acts locally /or is transported to other tissues via blood circulation. Testosterone synthesis takes place within the mitochondria of Leydig cells, the testosterone-producing cells of the testis. It is produced upon stimulation of these cells by Luteinizing hormone (LH) that is secreted in pulses into the peripheral circulation by the pituitary gland in response to Gonadotropin-releasing hormone (GnRH) from the hypothalamus. Testosterone and its aromatized product, estradiol, feed back to the hypothalamus and pituitary gland to suppress transiently LH and thus testosterone production. In response to reduced testosterone levels, GnRH and LH are produced. This negative feedback cycle results in pulsatile secretion of LH followed by pulsatile production of testosterone (Ellis, Desjardins, and Fraser 1983), (Chandrashekar and Bartke 1998).

General role in biology

Testosterone is the principal male sex hormone and an anabolic steroid. Male sexual differentiation depends on testosterone (T), dihydrotestosterone (DHT), and the expression of androgen receptors by target cells (Manson and Carr 2003). During the development secretion of androgens by Leydig cells is essential for masculinization of the foetus (Nef 2000). The foetal Leydig cells develop in utero. These cells become competent to produce testosterone in rat by gestational day (GD) 15.5, with increasing production thereafter. Peak steroidogenic activity is reached just prior to birth, on GD19 (Chen, Ge, and Zirkin 2009). Testosterone secreted by foetal Leydig cells is required for the differentiation of the male urogenital system late in gestation (Huhtaniemi and Pelliniemi 1992). Foetal Leydig cells also play a role in the scrotal descent of the testis through their synthesis of insulin-like growth factor 3 (Insl3), for review see (Nef 2000).

In humans, the first morphological sign of testicular differentiation is the formation of testicular cords, which can be seen between 6 and 7 weeks of gestation. Steroid-secreting Leydig cells can be seen in the testis at 8 weeks of gestation. At this period, the concentration of androgens in the testicular tissue and blood starts to rise, peaking at 14-16 weeks of gestation. This increase comes with an increase in the number of Leydig cells for review see (Rouiller-Fabre et al. 2009).

Adult Leydig cells, which are distinct from the foetal Leydig cells, form during puberty and supply the testosterone required for the onset of spermatogenesis, among other functions. Distinct stages of adult Leydig cell development have been identified and characterized. The stem Leydig cells are undifferentiated cells that are capable of indefinite self-renewal but also of differentiation to steroidogenic cells. These cells give rise to progenitor Leydig cells, which proliferate, continue to differentiate, and give rise to the immature Leydig cells. Immature Leydig cells synthesize high levels of testosterone metabolites and develop into terminally differentiated adult Leydig cells, which produce high levels of testosterone. With aging, both serum and testicular testosterone concentrations progressively decline, for review see (Nef 2000).

Androgens play a crucial role in the development and maintenance of male reproductive and sexual functions. Low levels of circulating androgens can cause disturbances in male sexual development, resulting in congenital abnormalities of the male reproductive tract. Later in life, this may cause reduced fertility, sexual dysfunction, decreased muscle formation and bone mineralisation, disturbances of fat metabolism, and cognitive dysfunction. Testosterone levels decrease as a process of ageing: signs and symptoms caused by this decline can be considered a normal part of ageing.

How it is Measured or Detected

OECD TG 456 [1] (http://www.oecd-ilibrary.org/environment/test-no-456-h295r-steroidogenesis-assay_9789264122642-en) is the validated test guideline for an in vitro screen for chemical effects on steroidogenesis, specifically the production of 17ß-estradiol (E2) and testosterone (T). The testosterone syntheis can be measured in vitro cultured Leydig cells. The methods for culturing Leydig cells can be found in the Database Service on Alternative Methods to animal experimentation (DB-ALM): Leydig Cell-enriched Cultures [2] (http://ecvam-dbalm.jrc.ec.europa.eu/beta/index.cfm/methodsAndProtocols/index?id_met=232), Testicular Organ and Tissue Culture Systems [3] (http://ecvam-dbalm.jrc.ec.europa.eu/beta/index.cfm/methodsAndProtocols/index?id_met=515).

Testosterone synthesis in vitro cultured cells can be measured indirectly by testosterone radioimmunoassay or analytical methods such as LC-MS.

References

Chandrashekar, V, and A Bartke. 1998. “The Role of Growth Hormone in the Control of Gonadotropin Secretion in Adult Male Rats.” Endocrinology 139 (3) (March): 1067–74. doi:10.1210/endo.139.3.5816.

Ellis, G B, C Desjardins, and H M Fraser. 1983. “Control of Pulsatile LH Release in Male Rats.” Neuroendocrinology 37 (3) (September): 177–83. Huhtaniemi, I, and L J Pelliniemi. 1992. “Fetal Leydig Cells: Cellular Origin, Morphology, Life Span, and Special Functional Features.” Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.) 201 (2) (November): 125–40.

Manson, Jeanne M, and Michael C Carr. 2003. “Molecular Epidemiology of Hypospadias: Review of Genetic and Environmental Risk Factors.” Birth Defects Research. Part A, Clinical and Molecular Teratology 67 (10) (October): 825–36. doi:10.1002/bdra.10084.

Nef, S. 2000. “Hormones in Male Sexual Development.” Genes & Development 14 (24) (December 15): 3075–3086. doi:10.1101/gad.843800.

Rouiller-Fabre, Virginie, Vincent Muczynski, Romain Lambrot, Charlotte Lécureuil, Hervé Coffigny, Catherine Pairault, Delphine Moison, et al. 2009. “Ontogenesis of Testicular Function in Humans.” Folia Histochemica et Cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society 47 (5) (January): S19–24. doi:10.2478/v10042-009-0065-4.

Event: 447: Reduction, Cholesterol transport in mitochondria (https://aopwiki.org/events/447)

Short Name: Reduction, Cholesterol transport in mitochondria

Key Event Component

Process	Object	Action
mitochondrial transport	cholesterol	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	KeyEvent
Aop:18 - PPARα activation in utero leading to impaired fertility in males (https://aopwiki.org/aops/18)	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
steroid hormone secreting cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)

The enzymes needed for cholesterol transport were found in amphioxus and are present in vertebrates (Albalat et al. 2011).

Key Event Description

Biological state

Steroidogenesis begins with the transport of cholesterol from intracellular stores into mitochondria. This process involves a series of protein-protein interactions involving cytosolic and mitochondrial proteins located at both the outer and inner mitochondrial membranes. In steroidogenic cells the cholesterol import to the mitochondrial inner membrane is crucial for steroid synthesis (Rone, Fan, and Papadopoulos 2009). This process is facilitated by the Scavenger Receptor Class B, type 1 (SR-B1) [more relevant for rodents, than for humans] that mediates the selective uptake of cholesterol esters from high-density lipoproteins. Steroidogenic acute regulatory protein (STAR) and the translator protein (TSPO) [former peripheral benzodiazepine receptor (PBR)] mediate cholesterol transport from the outer to the inner mitochondrial membrane. The conversion of cholesterol to pregnenolone is done by Cholesterol side-chain cleavage enzyme (P450scc), the start of steroidogenesis [reviewed in (Miller and Auchus 2011)].

Biological compartments

In mitochondria of steroidogenic tissues there are two specialized mechanisms related to hormone synthesis: one by which cholesterol is delivered to the mitochondria and the other by which specialized intra-mitochondrial enzymes participate in the synthesis of hormonal steroids.

General role in biology

Systemic steroid hormones are primarily formed by the gonads, adrenal glands, and during in utero development by the placenta. Some other organs like brain (Baulieu 1998), and heart (Kayes-Wandover and White 2000) have also been identified as steroid-producing tissues, mainly for local needs. The steroid hormones are indispensable for mammalian life. They are made from cholesterol via complex biosynthetic pathways that are initiated by specialized, tissue-specific enzymes in mitochondria. These hormones include glucocorticoids (cortisol, corticosterone) and mineralocorticoids (aldosterone) produced in the adrenal cortex, estrogens (estradiol), progestins (progesterone) and androgens (testosterone, dihydrotestosterone) produced in the gonads, and calciferols (1,25-dihydroxy vitamin D [1,25OH2D]) produced in the kidneys (Miller and Auchus 2011). Cholesterol is the precursor for the synthesis of steroid hormones in mitochondria. Steroidogenesis begins with the metabolism of cholesterol to pregnenolone facilitated by P450scc. The rate of steroid formation depends on the rate of cholesterol transport from intracellular stores to the inner mitochondrial membrane and the loading of P450scc with cholesterol (Miller and Auchus 2011). Interference with one or more of these reactions leads to reduced steroid production.

How it is Measured or Detected

This KE can be indirectly measured by:

1. Expression of the proteins involved in cholesterol transport by qPCR or Western blot.

3. Cholesterol transport to the mitochondrial inner membrane in intact cells:

- Indirectly as pregnenolone formation by cells. The pregnenolone concentration is assayed by commercially available radioimmunoassays and reflects the amount of cholesterol transported to the mitochondrial inner membrane (Charman et al. 2010).
- Filipin staining is one of the most widely used tools for studying intracellular cholesterol distribution. The fluorescent detergent filipin binds selectively to cholesterol (and not to cholesterol esters) (Schroeder, Holland, and Bieber 1971). Filipin can be only used for the qualitative analysis of cholesterol distribution, since its fluorescence intensity is not necessarily linearly related to cholesterol content.

The cholesterol transport can be measured *in vitro* cultured Leydig cells. The methods for culturing Leydig cells can be found in the Database Service on Alternative Methods to animal experimentation (DB-ALM): Leydig Cell-enriched Cultures [1] (http://ecvam-dbalm.jrc.ec.europa.eu/beta/index.cfm/methodsAndProtocols/index?id_met=232) Testicular Organ and Tissue Culture Systems [2] (http://ecvam-dbalm.jrc.ec.europa.eu/beta/index.cfm/methodsAndProtocols/index?id_met=515)

References

Albalat, Ricard, Frédéric Brunet, Vincent Laudet, and Michael Schubert. 2011. “Evolution of Retinoid and Steroid Signaling: Vertebrate Diversification from an Amphioxus Perspective.” Genome Biology and Evolution 3: 985–1005. doi:10.1093/gbe/evr084.

Baulieu, E E. 1998. “Neurosteroids: A Novel Function of the Brain.” Psychoneuroendocrinology 23 (8) (November): 963–87.

Charman, Mark, Barry E Kennedy, Nolan Osborne, and Barbara Karten. 2010. “MLN64 Mediates Egress of Cholesterol from Endosomes to Mitochondria in the Absence of Functional Niemann-Pick Type C1 Protein.” Journal of Lipid Research 51 (5) (May): 1023–34. doi:10.1194/jlr.M002345.

Kayes-Wandover, K M, and P C White. 2000. “Steroidogenic Enzyme Gene Expression in the Human Heart.” The Journal of Clinical Endocrinology and Metabolism 85 (7) (July): 2519–25. doi:10.1210/jcem.85.7.6663.

Miller, Walter L, and Richard J Auchus. 2011. “The Molecular Biology, Biochemistry, and Physiology of Human Steroidogenesis and Its Disorders.” Endocrine Reviews 32 (1) (February): 81–151. doi:10.1210/er.2010-0013.

Rone, Malena B, Jinjiang Fan, and Vassilios Papadopoulos. 2009. “Cholesterol Transport in Steroid Biosynthesis: Role of Protein-Protein Interactions and Implications in Disease States.” Biochimica et Biophysica Acta 1791 (7) (July): 646–58. doi:10.1016/j.bbalip.2009.03.001.

Schroeder, F, J F Holland, and L L Bieber. 1971. “Fluorometric Evidence for the Binding of Cholesterol to the Filipin Complex.” The Journal of Antibiotics 24 (12) (December): 846–9.

Steer, C. 1984. “Detection of Membrane Cholesterol by Filipin in Isolated Rat Liver Coated Vesicles Is Dependent upon Removal of the Clathrin Coat.” The Journal of Cell Biology 99 (1) (July 1): 315–319. doi:10.1083/jcb.99.1.315.

List of Adverse Outcomes in this AOP

Event: 406: impaired, Fertility (https://aopwiki.org/events/406)

Short Name: impaired, Fertility

Key Event Component

Process	Object	Action
fertility		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:7 - Aromatase (Cyp19a1) reduction leading to impaired fertility in adult female (https://aopwiki.org/aops/7)	AdverseOutcome
Aop:51 - PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	AdverseOutcome
Aop:18 - PPARα activation in utero leading to impaired fertility in males (https://aopwiki.org/aops/18)	AdverseOutcome
Aop:64 - Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility (https://aopwiki.org/aops/64)	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
mouse	Mus musculus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Key Event Description

Biological state

capability to produce offspring

Biological compartments

System

General role in biology

Fertility is the capacity to conceive or induce conception. Impairment of fertility represents disorders of male or female reproductive functions or capacity.

How it is Measured or Detected

As a measure, fertility rate, is the number of offspring born per mating pair, individual or population.

Regulatory Significance of the AO

Under REACH, information on reproductive toxicity is required for chemicals with an annual production/importation volume of 10 metric tonnes or more. Standard information requirements include a screening study on reproduction toxicity (OECD TG 421/422) at Annex VIII (10-100 t.p.a), a prenatal developmental toxicity study (OECD 414) on a first species at Annex IX (100-1000 t.p.a), and from March 2015 the OECD 443(Extended One-Generation Reproductive Toxicity Study) is reproductive toxicity requirement instead of the two generation reproductive toxicity study (OECD TG 416). If not conducted already at Annex IX, a prenatal developmental toxicity study on a second species at Annex X (≥ 1000 t.p.a.).

Under the Biocidal Products Regulation (BPR), information is also required on reproductive toxicity for active substances as part of core data set and additional data set (EU 2012, ECHA 2013). As a core data set, prenatal developmental toxicity study (EU TM B.31) in rabbits as a first species and a two-generation reproduction toxicity study (EU TM B.31) are required. OECD TG 443 (Extended One-Generation Reproductive Toxicity Study) shall be considered as an alternative approach to the multi-generation study.) According to the Classification, Labelling and Packaging (CLP) regulation (EC, 200; Annex I: 3.7.1.1): a) “reproductive toxicity” includes adverse effects on sexual function and fertility in adult males and females, as well as developmental toxicity in the offspring; b) “effects on fertility” includes adverse effects on sexual function and fertility; and c) “developmental toxicity” includes adverse effects on development of the offspring.

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 398: Increase proliferation, Leydig cell leads to Hyperplasia, Leydig cell (https://aopwiki.org/relationships/398)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	**adjacent**	**Moderate**	

Relationship: 439: Reduction, Testosterone synthesis in Leydig cells leads to Reduction, testosterone level (https://aopwiki.org/relationships/439)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
PPARα activation in utero leading to impaired fertility in males (https://aopwiki.org/aops/18)	**adjacent**	**High**	
PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	**adjacent**	**High**	
Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility (https://aopwiki.org/aops/64)	**adjacent**		

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
human	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)
mice	Mus sp.	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10095)

Ses Table 1.

Key Event Relationship Description

Impairment of testosterone production in testes directly impacts on testosterone levels.

Evidence Supporting this KER

Biological Plausibility

Within the testes, steroid synthesis takes place within the mitochondria of Leydig cells. Testosterone production by Leydig cells is primarily under the control of LH. LH indirectly stimulates the transfer of cholesterol into the mitochondrial matrix to cholesterol side-chain cleavage cytochrome P450 (P450scc, CYP11A), which converts cholesterol to pregnenolone. Pregnenolone diffuses to the smooth endoplasmic reticulum where it is further metabolized to testosterone via the actions of 3β-

hydroxysteroid dehydrogenase Δ5-Δ4-isomerase (3β-HSD), 17α-hydroxylase/C17-20 lyase (P450c17, CYP17), and 17β-hydroxysteroid dehydrogenase type III (17HSD3). For review see (Payne & Hales, 2013). Therefore, inhibition or impairment of the testosterone production directly impacts on the levels of testosterone.

Empirical Evidence

There is evidence from experimental work that demonstrates a coordinated, dose-dependent reduction in the production of testosterone and consecutive reduction of testosterone levels in foetal testes and in serum, see Table 1.

			KE: testosterone synthesis, reduction	**KE: testosterone, reduction**		
Compound	Species	Effect level			Details	References
Phthalates (DEHP)	rat	LOEL =300 mg/kg/day	testicular testosterone production, reduction (ex vivo)	testicular testosterone levels, reduction, no change plasma testosterone	testosterone levels at GD 21 in male rat fetuses exposed to 0, 10, 30, 100, or 300 mg /kg bw/day from GD 7 to GD 21 testicular testosterone production ex vivo	(Borch, Metzdorff, Vinggaard, Brokken, & Dalgaard, 2006)
Phthalates (DBP)	rat	LOEL =50 mg/kg/day		testicular testosterone levels, reduction,	Testicular testosterone was reduced >50 mg/kg/day	(Shultz, 2001)
Phthalates (DEHP)	rat	LOEL=300 mg/kg/day	fetal testicular testosterone production, reduction			(Borch, Ladefoged, Hass, & Vinggaard, 2004)
Phthalates (DEHP)	rat	LOEL=300 mg/kg/day		testicular testosterone levels, reduction,		(Borch et al., 2004)
Phthalates (DEHP)	rat	LOEL=300 mg/kg/day		No change plasma testosterone		(Borch et al., 2004)
Phthalates (DEHP)	rat	LOEL=100 mg/kg/day		Serum testosterone levels, reduction,		(Akingbemi, 2001)
Phthalates (DEHP)	rat	LOEL=750 mg /kg /day		testicular testosterone levels, reduction, by 60 – 85%		(Parks, 2000)
Phthalates (DEHP)	rat	LOEL=750 mg /kg/day		testosterone levels, reduction, fetuses on GD 17 (71% lower than controls) and 18 (47% lower than controls)		(Parks, 2000)
Phthalates (DEHP)	rat	LOEL=750mg/kg/day	ex vivo testosterone production, reduction by 50%			(Wilson et al., 2004)
Phthalates (DEHP)	rat	LOEL=234 mg/kg/day		serum testosterone levels, reduction,		(Culty et al., 2008)
Phthalates (DEHP)	rat	LOEL=1250 mg/kg/day	*ex vivo* foetal testicular production			(Culty et al., 2008)
Phthalates (DEHP)	rat	ED50=444,2 mg/kg/day	*ex vivo* foetal testicular production, reduction			(Hannas et al., 2012)
Phthalates (DHP)	rat	ED50=75.25 mg/kg/day	*ex vivo* foetal testicular production, reduction			(Hannas et al., 2012)

Table 1. Summary table for empirical support for this KER. ED50 - half maximal effective concentration, LOEL- lowest observed effect level, Dibutyl phthalate (DBP), Bis(2-ethylhexyl) phthalate (DEHP), Dihexyl Phthalate (DHP).

References

Akingbemi, B. T. 2001. “Modulation of Rat Leydig Cell Steroidogenic Function by Di(2-Ethylhexyl)Phthalate.” Biology of Reproduction 65 (4) (October 1): 1252–1259. doi:10.1095/biolreprod65.4.1252.

Borch, Julie, Ole Ladefoged, Ulla Hass, and Anne Marie Vinggaard. 2004. “Steroidogenesis in Fetal Male Rats Is Reduced by DEHP and DINP, but Endocrine Effects of

DEHP Are Not Modulated by DEHA in Fetal, Prepubertal and Adult Male Rats." Reproductive Toxicology (Elmsford, N.Y.) 18 (1): 53–61. doi:10.1016/j.reprotox.2003.10.011.

Borch, Julie, Stine Broeng Metzdorff, Anne Marie Vinggaard, Leon Brokken, and Majken Dalgaard. 2006. "Mechanisms Underlying the Anti-Androgenic Effects of Diethylhexyl Phthalate in Fetal Rat Testis." Toxicology 223 (1-2) (June 1): 144–55. doi:10.1016/j.tox.2006.03.015.

Culty, Martine, Raphael Thuillier, Wenping Li, Yan Wang, Daniel B Martinez-Arguelles, Carolina Gesteira Benjamin, Kostantinos M Triantafilou, Barry R Zirkin, and Vassilios Papadopoulos. 2008. "In Utero Exposure to Di-(2-Ethylhexyl) Phthalate Exerts Both Short-Term and Long-Lasting Suppressive Effects on Testosterone Production in the Rat." Biology of Reproduction 78 (6) (June): 1018–28. doi:10.1095/biolreprod.107.065649.

Hannas, Bethany R, Christy S Lambright, Johnathan Furr, Nicola Evans, Paul M D Foster, Earl L Gray, and Vickie S Wilson. 2012. "Genomic Biomarkers of Phthalate-Induced Male Reproductive Developmental Toxicity: A Targeted RT-PCR Array Approach for Defining Relative Potency." Toxicological Sciences : An Official Journal of the Society of Toxicology 125 (2) (February): 544–57. doi:10.1093/toxsci/kfr315.

Parks, L. G. 2000. "The Plasticizer Diethylhexyl Phthalate Induces Malformations by Decreasing Fetal Testosterone Synthesis during Sexual Differentiation in the Male Rat." Toxicological Sciences 58 (2) (December 1): 339–349. doi:10.1093/toxsci/58.2.339.

Shultz, V. D. 2001. "Altered Gene Profiles in Fetal Rat Testes after in Utero Exposure to Di(n-Butyl) Phthalate." Toxicological Sciences 64 (2) (December 1): 233–242. doi:10.1093/toxsci/64.2.233.

Wilson, Vickie S., Christy Lambright, Johnathan Furr, Joseph Ostby, Carmen Wood, Gary Held, and L.Earl Gray. 2004. "Phthalate Ester-Induced Gubernacular Lesions Are Associated with Reduced insl3 Gene Expression in the Fetal Rat Testis." Toxicology Letters 146 (3) (February): 207–215. doi:10.1016/j.toxlet.2003.09.012.

Relationship: 438: Reduction, Cholesterol transport in mitochondria leads to Reduction, Testosterone synthesis in Leydig cells (https://aopwiki.org/relationships/438)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
PPARα activation in utero leading to impaired fertility in males (https://aopwiki.org/aops/18)	**adjacent**	**Moderate**	
PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	**adjacent**	**Moderate**	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mice	Mus sp.	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10095)
rat	Rattus norvegicus	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10116)
human	Homo sapiens	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

See Table 1.

Key Event Relationship Description

Production of steroid hormones depends on the availability of cholesterol in the mitochondrial matrix. A decreased amount of cholesterol inside the mitochondria (e. g by decreased expression of enzymes that transport cholesterol like StAR or TSOP) means diminished substrate for hormone (testosterone) production in testes.

Evidence Supporting this KER

Biological Plausibility

Steroid hormones play a critical role in sexual development, homeostasis, stress-responses, carbohydrate metabolism, tumor growth, and reproduction. These hormones are primarily produced in specialized steroidogenic tissues and are synthesized from a common precursor, cholesterol. Mitochondria are a key control point for the regulation of steroid hormone biosynthesis. The first and rate-limiting step in steroidogenesis is the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, a process dependent on the action of StAR (Stocco, 2001) and the subsequent transport across the inner mitochondrial space into the steroidogenic pathway, which is executed by TSPO (Hauet et al., 2005). Testosterone production by Leydig cells is primarily under the control of luteinizing hormone (LH). Stimulation of the Leydig cells results in the activation of StAR transcription and translation, which facilitates the transfer of cholesterol into the mitochondrial matrix to cholesterol side-chain cleavage cytochrome P450 (P450scc, CYP11A), which converts cholesterol to pregnenolone. Pregnenolone diffuses to the smooth endoplasmic reticulum where it is further metabolized to testosterone via the actions of 3β-hydroxysteroid dehydrogenase Δ5-Δ4-isomerase (3β-HSD), 17α-hydroxylase/C17-20 lyase (P450c17, CYP17), and 17β-hydroxysteroid dehydrogenase type III (17HSD3). For review see (Payne & Hales, 2013). Decreased expression of genes that are responsible for cholesterol transport and steroidogenic enzyme activities in the Leydig cell leads to decreased testosterone production.

Empirical Evidence

There is evidence from experimental work that demonstrates a coordinated reduction in the expression of key genes and proteins that are involved in cholesterol transport and steroidogenesis, together with a corresponding reduction in testosterone in testes. For details see Table 1. Foetal Leydig cells exhibit a high rate of lipid metabolism, which is required for both synthesizing and importing the testosterone precursor cholesterol. Upon exposure to some chemicals mRNA expression of genes in these pathways are profoundly reduced e.g. following 500mg/kg phthalate (DBP) exposure (Johnson, McDowell, Viereck, & Xia, 2011), (Thompson et al., 2005). Additionally, after phthalate exposure testis cholesterol and cholesterol-containing lipid droplets in foetal Leydig cells are also reduced (Barlow et al., 2003), (Johnson et al., 2011), (Lehmann, Phillips, Sar, Foster, & Gaido, 2004).

			KE: Cholesterol transport, reduction	**KE: Testosterone production/levels, reduction**

Compound	Species	Effect level	Translator protein (TSPO), decrease; Steroidogenic acute regulatory protein (StAR) decrease	
Phthalate (DBP)	rat	LOEL=500 mg/kg/day	mRNA StAR decrease (by ~34%) (Barlow et al., 2003)	
Phthalate (BBP, DPeP, DEHP, DHP, DiHeP, DCHP, DINP DHeP)	rat			
Phthalate (DBP, DEHP, BBP)	Rat	LOEL=750 mg/kg/day (GD14-18)		testosterone production, reduction ex vivo fetal testes examined on GD18 (Wilson et al., 2004)
Phthalate (DBP)	rat	LOEL=500 mg/kg/day	reduced Leydig cell lipid content(Barlow et al., 2003)	
Phthalate (DBP)	rat	LOEL=500 mg/kg/day GD 12 -20, examinations on GD20	total cholesterol levels, reduction	intratesticular testosterone levels, reduction (by nearly 90%) (Johnson et al., 2011)
Phthalate (DBP)	rat	LOEL=500 mg/kg/day (GD12-19)	decrease uptake of cholesterol Leydig cell mitochondria gd 19	testosterone production, reduction ex vivo (Thompson, Ross, & Gaido, 2004)
Phthalate (DEHP)	mouse	LOEL=1 g/kg/day	reduced TSPO mRNA	testosterone levels, reduction (Gazouli, 2002)
Phthalate (DEHP)	rat	LOEL=300 mg/ kg/day	dose-dependently reduced StAR, TSOP mRNA (GD 21 testes), also on protein levels in Leydig cells (Borch, Metzdorff, Vinggaard, Brokken, & Dalgaard, 2006)	
Phthalate (DEHP)	rat	LOEL=300 mg/kg/day		testosterone production, reduction (ex vivo) testosterone levels, reduction (Borch et al., 2006), (Borch, Ladefoged, Hass, & Vinggaard, 2004)
Phthalate (MEHP)	mouse	LOEC=100 µM	• reduced TSPO mRNA levels by 50%, • binding sites decreased by 50% • no effect on receptor affinity • inhibited the transfer or loading of cholesterol to the inner mitochondrial membrane P450scc. (Gazouli, 2002)	
Phthalate (MEHP)	rat	IC50 =100 µM	• inhibited formation of progesterone (Gazouli, 2002)	
Phthalate (MEHP)	rat	LOEC=250 µM	cholesterol transport, decrease (into the mitochondria of immature and adult Leydig cells)	Testosterone, reduction by approximately 60%, in vitro (immature and adult Leydig cells) (Svechnikov, Svechnikova, & Söder, 2008)
Phthalate (DEHP)	rat	LOEL=750 mg/kg/day		testosterone production reduction, testosterone levels, reduction (testicular and whole-body T levels in fetal and neonatal male rats from GD 17 to PND 2. (Parks, 2000)
Phthalate (MEHP)	rat	LOEC=1 µM		testosterone production, reduction dose-dependent (Chauvigné et al., 2011)
Perfluorooctanoic acid (PFOA)	mouse	LOEL=5mg/kg/day		plasma testosterone, reduction (by 37%)(Li et al., 2011)
WY-14,643	mouse	LOEC=50 mg/kg/day	reduced TSPO mRNA	Serum testosterone levels, reduction (Gazouli, 2002)
WY-14,643	rat			No decrease of testosterone (ex vivo), (Furr, Lambright, Wilson, Foster, & Gray, 2014)
WY-14,643	mouse	LOEC=100 µM	Inhibited progesterone synthesis (Gazouli, 2002)	

Bezafibrate	mouse	IC50=100 μM	• a dose-dependent 10–95% inhibition of the progesterone synthesis at 24 or 72 h • inhibited the transfer or loading of cholesterol to the inner mitochondrial membrane P450scc. At 100 μM • binding sites of TSPO decreased IC50 of approximately 100 μM • decrease TSPO levels by 60% at 100 μM (Gazouli, 2002)	
Bezafibrate	rat	IC50 = 30 μM	inhibited formation of progesterone (Gazouli, 2002)	
Bezafibrate	rat	IC50 ~10–4 μM		testosterone production, reduction (Gazouli, 2002)
Phthalate (DiBP)	rat	GD 19 -21	reduced StAR, (Boberg et al., 2008)	testicular testosterone production and testicular testosterone levels, (Boberg et al., 2008)

Table 1. Summary table of empirical support for this KER. IC50 half maximal inhibitory concentration, LOEC-lowest effect concentration, LOEL- lowest observed effect level, Dibutyl phthalate (DBP), diisobutyl phthalate (DiBP), Bis(2-ethylhexyl) phthalate (DEHP), Dibutyl phthalate (DBP), Bezafibrate and WY-14,643 are PPARα ligands, n.a - not available

Uncertainties and Inconsistencies

Thompson et al investigated time course effects of phthalate on steroidogenesis gene expression and testosterone concentration. The study showed diminished concentration testosterone concentration in the foetal testis by 50% within 1h of treatment with phthalate (DBP). Surprisingly, the diminution in testosterone concentration preceded any alteration in expression of genes in the steroidogenesis pathway. Star mRNA was significantly diminished 2 h after DBP exposure, but Cyp11a1, Cyp17a1, and Scarb1 did not show a significant decrease in expression until 6 h after DBP exposure (Thompson et al., 2005). In utero exposure of rats to PFOA 20 mg/kg did not cause any effect on fetal testosterone (Boberg et.al. 2008) although in mice (adult) the decrease level of testosterone was observed. Testosterone production may also be diminished due to reduction/inhibition of other genes involved in steroidogenesis (e.g. P450scc, Cyp17a1) (Thompson et al., 2004), (Boberg et al., 2008), (Chauvigné et al., 2009), (Chauvigné et al., 2011).

References

Barlow, N. J., Phillips, S. L., Wallace, D. G., Sar, M., Gaido, K. W., & Foster, P. M. D. (2003). Quantitative changes in gene expression in fetal rat testes following exposure to di(n-butyl) phthalate. Toxicological Sciences : An Official Journal of the Society of Toxicology, 73(2), 431–41. doi:10.1093/toxsci/kfg087

Boberg, J., Metzdorff, S., Wortziger, R., Axelstad, M., Brokken, L., Vinggaard, A. M., … Nellemann, C. (2008). Impact of diisobutyl phthalate and other PPAR agonists on steroidogenesis and plasma insulin and leptin levels in fetal rats. Toxicology, 250(2-3), 75–81. doi:10.1016/j.tox.2008.05.020

Borch, J., Ladefoged, O., Hass, U., & Vinggaard, A. M. (2004). Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats. Reproductive Toxicology (Elmsford, N.Y.), 18(1), 53–61. doi:10.1016/j.reprotox.2003.10.011

Borch, J., Metzdorff, S. B., Vinggaard, A. M., Brokken, L., & Dalgaard, M. (2006). Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis. Toxicology, 223(1-2), 144–55. doi:10.1016/j.tox.2006.03.015

Chauvigné, F., Plummer, S., Lesné, L., Cravedi, J.-P., Dejucq-Rainsford, N., Fostier, A., & Jégou, B. (2011). Mono-(2-ethylhexyl) phthalate directly alters the expression of Leydig cell genes and CYP17 lyase activity in cultured rat fetal testis. PloS One, 6(11), e27172. doi:10.1371/journal.pone.0027172

Furr, J. R., Lambright, C. S., Wilson, V. S., Foster, P. M., & Gray, L. E. (2014). A short-term in vivo screen using fetal testosterone production, a key event in the phthalate adverse outcome pathway, to predict disruption of sexual differentiation. Toxicological Sciences : An Official Journal of the Society of Toxicology, 140(2), 403–24. doi:10.1093/toxsci/kfu081

Gazouli, M. (2002). Effect of Peroxisome Proliferators on Leydig Cell Peripheral-Type Benzodiazepine Receptor Gene Expression, Hormone-Stimulated Cholesterol Transport, and Steroidogenesis: Role of the Peroxisome Proliferator-Activator Receptor . Endocrinology, 143(7), 2571–2583. doi:10.1210/en.143.7.2571

Hauet, T., Yao, Z.-X., Bose, H. S., Wall, C. T., Han, Z., Li, W., … Papadopoulos, V. (2005). Peripheral-type benzodiazepine receptor-mediated action of steroidogenic acute regulatory protein on cholesterol entry into leydig cell mitochondria. Molecular Endocrinology (Baltimore, Md.), 19(2), 540–54. doi:10.1210/me.2004-0307

Johnson, K. J., McDowell, E. N., Viereck, M. P., & Xia, J. Q. (2011). Species-specific dibutyl phthalate fetal testis endocrine disruption correlates with inhibition of SREBP2-dependent gene expression pathways. Toxicological Sciences : An Official Journal of the Society of Toxicology, 120(2), 460–74. doi:10.1093/toxsci/kfr020

Lehmann, K. P., Phillips, S., Sar, M., Foster, P. M. D., & Gaido, K. W. (2004). Dose-dependent alterations in gene expression and testosterone synthesis in the fetal testes of male rats exposed to di (n-butyl) phthalate. Toxicological Sciences : An Official Journal of the Society of Toxicology, 81(1), 60–8. doi:10.1093/toxsci/kfh169

Li, Y., Ramdhan, D. H., Naito, H., Yamagishi, N., Ito, Y., Hayashi, Y., … Nakajima, T. (2011). Ammonium perfluorooctanoate may cause testosterone reduction by adversely affecting testis in relation to PPARα. Toxicology Letters, 205(3), 265–72. doi:10.1016/j.toxlet.2011.06.015 Miller, W. L. (2007). Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter. Biochimica et Biophysica Acta, 1771(6), 663–76. doi:10.1016/j.bbalip.2007.02.012

Parks, L. G. (2000). The Plasticizer Diethylhexyl Phthalate Induces Malformations by Decreasing Fetal Testosterone Synthesis during Sexual Differentiation in the Male Rat. Toxicological Sciences, 58(2), 339–349. doi:10.1093/toxsci/58.2.339

Payne, A. H., & Hales, D. B. (2013). Overview of Steroidogenic Enzymes in the Pathway from Cholesterol to Active Steroid Hormones. Endocrine Reviews. Stocco, D. M. (2001). StAR protein and the regulation of steroid hormone biosynthesis. Annual Review of Physiology, 63, 193–213. doi:10.1146/annurev.physiol.63.1.193

Svechnikov, K., Svechnikova, I., & Söder, O. (2008). Inhibitory effects of mono-ethylhexyl phthalate on steroidogenesis in immature and adult rat Leydig cells in vitro. Reproductive Toxicology (Elmsford, N.Y.), 25(4), 485–90. doi:10.1016/j.reprotox.2008.05.057

Thompson, C. J., Ross, S. M., & Gaido, K. W. (2004). Di(n-butyl) phthalate impairs cholesterol transport and steroidogenesis in the fetal rat testis through a rapid and reversible mechanism. Endocrinology, 145(3), 1227–37. doi:10.1210/en.2003-1475

Thompson, C. J., Ross, S. M., Hensley, J., Liu, K., Heinze, S. C., Young, S. S., & Gaido, K. W. (2005). Differential steroidogenic gene expression in the fetal adrenal gland versus the testis and rapid and dynamic response of the fetal testis to di(n-butyl) phthalate. Biology of Reproduction, 73(5), 908–17. doi:10.1095/biolreprod.105.042382

Wilson, V. S., Lambright, C., Furr, J., Ostby, J., Wood, C., Held, G., & Gray, L. E. (2004). Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. Toxicology Letters, 146(3), 207–15.

List of Non Adjacent Key Event Relationships

Relationship: 460: Reduction, testosterone level leads to Increase, Luteinizing hormone (LH) (https://aopwiki.org/relationships/460)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	**non-adjacent**	**Moderate**	

Relationship: 461: Increase, Luteinizing hormone (LH) leads to Increase proliferation, Leydig cell (https://aopwiki.org/relationships/461)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	**non-adjacent**	**Moderate**	

Relationship: 462: Hyperplasia, Leydig cell leads to impaired, Fertility (https://aopwiki.org/relationships/462)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
PPARα activation leading to impaired fertility in adult male rodents (https://aopwiki.org/aops/51)	**non-adjacent**	**Moderate**	