AOP ID and Title:

SNAPSHOT

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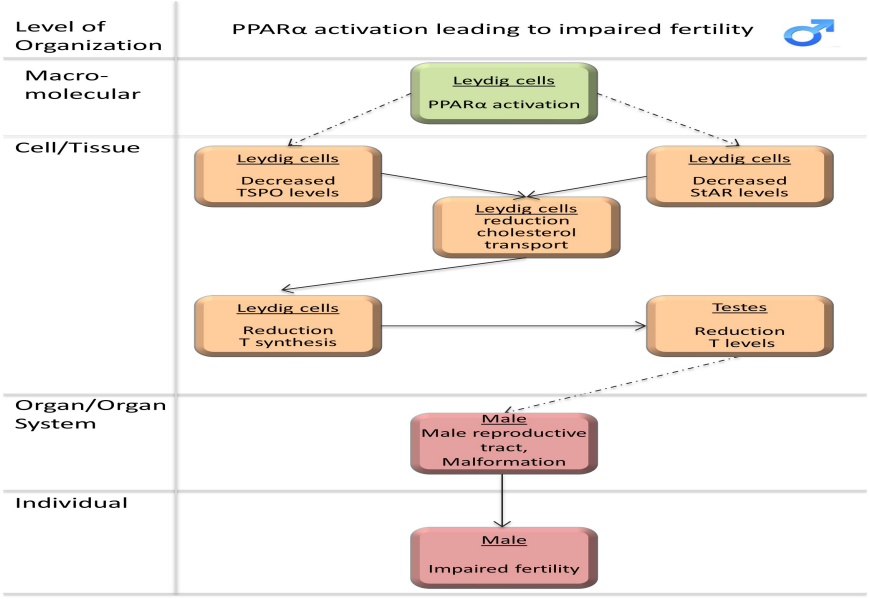
AOP 18: PPARα activation in utero leading to impaired fertility in males

Short Title: PPARα activation leading to impaired fertility

Graphical Representation



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Status

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Open for citation & comment	EAGMST Under Review	1.21	Included in OECD Work Plan

Abstract

This AOP links the activation of Peroxisome Proliferator Activated Receptor α (PPARα) to the developmental/reproductive toxicity in male. The development of this AOP relies on evidence collected from rodent models and incorporates human mechanistic and epidemiological data. The PPARα is a ligand-activated transcription factor that belongs to the nuclear receptor family, which also includes the steroid and thyroid hormone receptors. The hypothesis that PPARα action is the mechanistic basis for effects on the reproductive system arises from limited experimental data indicating relationships between activation of this receptor and impairment of steroidogenesis leading to reproductive toxicity. PPARs play important roles in the metabolic regulation of lipids, of which cholesterol, in particular, being a precursor of steroid hormones, makes the link between lipid metabolism to effects on reproduction. The key events in the pathway comprise the activation of PPARα, followed by the disruption cholesterol transport in mitochondria, impairment of hormonal balance which leads to malformation of the reproductive tract in males which may lead to impaired fertility. The PPARα-initiated AOP to rodent male developmental toxicity is a first step for structuring current knowledge about a mode of action which is neither AR-mediated nor via direct steroidogenesis enzymes inhibition. In the current form the pathway lays a strong basis for linking an endocrine mode of action with an apical endpoint, a prerequisite requirement for the identification of endocrine disrupting chemicals. This AOP is complemented with a structured data collection which will serve as the basis for further quantitative development of the pathway.

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	266	Decrease, Steroidogenic acute regulatory protein (STAR) (https://aopwiki.org/events/266)	Decrease, Steroidogenic acute regulatory protein (STAR)
3	KE	447	Reduction, Cholesterol transport in mitochondria (https://aopwiki.org/events/447)	Reduction, Cholesterol transport in mitochondria
4	KE	413	Reduction, Testosterone synthesis in Leydig cells (https://aopwiki.org/events/413)	Reduction, Testosterone synthesis in Leydig cells
5	KE	446	Reduction, testosterone level (https://aopwiki.org/events/446)	Reduction, testosterone level
6	KE	289	Decrease, Translocator protein (TSPO) (https://aopwiki.org/events/289)	Decrease, Translocator protein (TSPO)
7	AO	406	impaired, Fertility (https://aopwiki.org/events/406)	impaired, Fertility
8	AO	348	Malformation, Male reproductive tract (https://aopwiki.org/events/348)	Malformation, Male reproductive tract

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Decrease, Steroidogenic acute regulatory protein (STAR) (https://aopwiki.org/relationships/436)	adjacent	Reduction, Cholesterol transport in mitochondria	Moderate	
Reduction, Cholesterol transport in mitochondria (https://aopwiki.org/relationships/438)	adjacent	Reduction, Testosterone synthesis in Leydig cells	Moderate	
Reduction, Testosterone synthesis in Leydig cells (https://aopwiki.org/relationships/439)	adjacent	Reduction, testosterone level	High	
Decrease, Translocator protein (TSPO) (https://aopwiki.org/relationships/437)	adjacent	Reduction, Cholesterol transport in mitochondria	Low	
Malformation, Male reproductive tract (https://aopwiki.org/relationships/405)	adjacent	impaired, Fertility	High	
Activation, PPARα (https://aopwiki.org/relationships/369)	non-adjacent	Decrease, Steroidogenic acute regulatory protein (STAR)	Moderate	
Activation, PPARα (https://aopwiki.org/relationships/370)	non-adjacent	Decrease, Translocator protein (TSPO)	Low	
Reduction, testosterone level (https://aopwiki.org/relationships/608)	non-adjacent	Malformation, Male reproductive tract	High	

Overall Assessment of the AOP

Biological plausibility, coherence, and consistency of the experimental evidence

In the presented AOP it is hypothesized that the key events occur in a biologically plausible order prior to the development of adverse outcomes. The PPARα activators have been shown to alter steroidogenesis and impair reproduction [see reviews (Corton and Lapinskas 2004), (Latini et al. 2008), (David 2006)]. However, there are some conflicting reports on the involvement of PPARα as MIE of the proposed AOP (Johnson, Heger, and Boekelheide 2012), (David 2006). The biochemistry of steroidogenesis and the predominant role of the gonad in synthesis of the sex steroids are well established. Steroidogenesis is a complex process that is dependent on the availability of cholesterol in mitochondria. Perturbation of genes responsible for cholesterol transport and steroidogenic enzyme activities in the Leydig cell will lead to a decrease in testicular testosterone (T) production. As a consequence, androgen-dependent tissue differentiation/development is adversely affected. The physical manifestation of this event may be reproductive tract malformation and possibly leads to impaired fertility.

Concordance of dose-response relationships

This is a qualitative description of the pathway; the currently available studies provide quantitative information on dose-response relationships only partially. Experimental data are based on exposure to phthalates and indicate that key events of this pathway occur at similar dose levels. The effects of altered gene expression levels that are responsible for the cholesterol transport into the Leydig cells were shown at >50 mg/kg/bw, a dose at which foetal T was decreased and anatomical malformations (hypospadias) were produced (Mylchreest, Cattley, and Foster 1998), (Mylchreest 2000), (Akingbemi 2001), (Lehmann et al. 2004). Tailored experiments are required for the exploration of quantitative linkages.

Temporal concordance among the key events and the adverse outcome

This AOP bridges two life stages: the AOs are results of the chemical exposure during a critical prenatal period for male development, the masculinization programming window (MPW), within which androgens must act to ensure the correct development of the male reproductive tract (Welsh et al. 2008). Therefore, the AOP focuses on the exposures within the MPW (15.5–18.5 GD days in rats). The temporal relationship of exposure to gestation day has been investigated using phthalates and it has been demonstrated that the gestational timing of exposure is important for the production of the adverse effects on the male reproductive tract (reviewed in (Ema 2002)).

Moreover, the temporal relationship between alterations of gene expression and changes in testosterone production has been investigated for phthalates (DBP) (Lehmann et al. 2004), (Thompson et al. 2005). Initial increases in gene expression are followed by decreases in the expression of genes which are associated with steroidogenesis. The observed decreased steroidogenesis and subsequent decrease in testosterone levels is well established as precursors to anatomical changes in the developing male reproductive tract. Thus, those key events of gene expression are temporally consistent with subsequent events, however complete temporal concordance studies are missing.

Strength, consistency, and specificity of association of adverse effect and initiating event

The strength of the chosen chemical initiators as PPARα activators was shown to partially correlate with their ability to act as a male reproductive toxicant (Corton and Lapinskas 2004). The presented key events leading to a decrease in steroidogenesis are plausible and consistent with the observed effects. There is coherence between decreased testosterone synthesis and malformations.

Alternative mechanism(s) or MIE(s) described which may contribute/synergise the postulated AOP

The inhibitory effect of PPARα activation seems to be attributable to an impairment of the multistep process of cholesterol mobilization, transport into mitochondria, and steroidogenesis leading to impaired androgens production. Therefore, it is plausible that several other mechanisms may contribute to/synergise with this AOP. For example, activation of other isoforms of PPARs (PPARβ/δ or/and γ) is hypothesised to be relevant for the pathway (Lapinskas et al. 2005), (Shipley and Waxman 2004).

PPARγ activation

Opposing effects of PPARγ ligands (thiazolidinediones, TZD) on androgen levels and/or production in male humans (Dunaif et al. 1996), (Bloomgarden, Futterweit, and Poretsky 2001), (Vierhapper, Nowotny, and Waldhäusl 2003) and animal models have been described (Kempná et al. 2007), (Gasic et al. 1998), (Mu et al. 2000), (Arlt, Auchus, and Miller 2001), (Minge, Robker, and Norman 2008), (Gasic et al. 2001), (Veldhuis, Zhang, and Garmey 2002). In rats no effects of PPARγ ligand (rosiglitazone) on production or total circulating testosterone levels were seen (Boberg et al. 2008), however a decrease in basal or induced testosterone production occurred in the Leydig cells of rosiglitazone-treated rats (Couto et al. 2010).

Moreover, there are contradicting reports as to the presence of PPARγ in the foetal testes (Hannas et al. 2012). Few others transcription factors involved in regulation of lipid metabolism are hypothesized to mediate effects on fetal Leydig cell gene expression like sterol regulatory element–binding protein (SREBP) (Lehmann et al. 2004), (Shultz 2001), CCAAT/enhancer-binding protein-β (CEBPB) (Kuhl, Ross, and Gaido 2007) or NR5A1 (also known as steroidogenic factor 1; Sf1) (Borch et al. 2006). The downstream effects in the pathway might be due to the constellation of earlier events in fetal Leydig cells leading to decrease testosterone production and connected adverse outcomes. Alternative/synergistic MIEs relating to this pathway are hypothesised in the KER description. At present there are no strong views on the other possible MIEs.

Uncertainties, inconsistencies and data gaps

The major uncertainty in this AOP is the functional relationship between (MIE) PPARα activation leading to cholesterol transport reduction; possible mechanisms have been proposed but strong experimental support is missing and some conflicting data are reported. The dose response data to support this relationship are lacking. Studies exploring the role of PPARα using PPARα knockout mice showed that prenatal exposure to phthalates caused developmental malformations in both wild-type and PPARα knockout mice, thus suggesting a PPARα-independent mechanism. However, it is difficult to draw any conclusion on the role of PPARα in phthalate-related reproductive toxicity since the intrauterine administration of phthalate (DEHP) occurred before the critical period of reproductive tract differentiation (Peters et al. 1997). Intrauterine DEHP-treated PPARα-deficient mice, developed delayed testicular, renal and developmental toxicities, but no liver toxicity, compared to wild types, thus confirming the early observation by Lee et al. about the PPARα dependence of liver response and, more importantly, indicating that DEHP may induce reproductive toxicity through both PPARα-dependent and -independent mechanism (Ward et al. 1998). PPARα-independent reproductive toxicity observed by Ward et al. may conceivably be mediated by other PPAR isoforms, such as PPARβ and PPARγ, or by a non-receptor-mediated organ-specific mechanism (Barak et al. 1999). Other studies showed that the administration of DEHP resulted in milder testis lesions and higher testosterone levels in PPARα-null mice than in wild-type mice (Gazouli 2002). A more recent report, investigating the role of PPARα, showed decreased testosterone levels in PPARα(–/–) null control mice, suggesting a positive constitutive role for PPARα in maintaining Leydig cell steroid formation (Borch et al. 2006).

Inconsistencies Genomic studies by Hannas et al., demonstrated that PPARα agonist Wy-14,643, did not reduce foetal testicular testosterone production following gestational day 14–18 exposure, suggesting that the antiandrogenic activity of phthalates is not PPARα mediated (Hannas et al. 2012). Similarly, recent report by Furr et al. did not observe testosterone decrease after administration of Wy-14,643 in rat (ex vivo) (Furr et al. 2014).

Data Gaps: Complete/pathway driven studies to investigate the effects of PPARs and their role in male reproductive development are lacking. For establishing a solid quantitative and temporal coherent linkage, mode of action framework analysis for PPAR α mediated developmental toxicity are needed. This approach has been applied for the involvement of PPAR α in liver toxicity (Corton et al. 2014), (Wood et al. 2014).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Development	High

Taxonomic Applicability

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

Sex Applicability

Sex	Evidence
Male	High

Empirical information on dose-response relationships between the KEs, are not available, however there are solid empirical data that would inform a computational, predictive model for reproductive toxicity via PPARα activation.

Life Stage Applicability

This AOP is relevant for developing (prenatal) male.

Taxonomic Applicability

The experimental support for the pathway is mainly based on the animal (rat studies). Conflicting reports comes from the studies on mouse. Studies in mice report contradictory results. Recently, studies by Furr et al revealed that fetal T production can be inhibited by exposure to a phthalates in utero (CD-1 mice), but at a higher dose level than required in rats and causing systemic effects (Furr et al. 2014). However there are some earlier reports that chronic dietary administration of phthalates produces adverse testicular effects and reduces fertility in CD-1 mice (Heindel et al. 1989)

Sex Applicability

This AOP applies to males only.

Essentiality of the Key Events

KRs	Essentiality - KEs	level of confidence
PPAR alpha, Activation	PPAR alpha activation was found to indirectly alter the expression of genes involved in cholesterol transport in mitochondria	very weak
TSPO; StAR decrease	Alterations in the amount of cholesterol transport proteins in mitochondria impact on the levels of substrate for steroid hormones production.	weak
cholesterol transport in mitochondria, reduction	Production of steroid hormones depends on the availability of cholesterol to the enzymes in the mitochondrial matrix. Decreasing the amount of cholesterol inside the mitochondria will result in a diminished amount of substrate for hormone (testosterone) synthesis.	moderate
Testosterone synthesis, reduction	The gonads are generally considered the major source of circulating androgens. Consequently, if testosterone synthesis by testes is reduced, testosterone concentrations would be expected to decrease unless there are concurrent reductions in the rate of T catabolism.	strong
Testosterone, reduction	Male sexual differentiation in general depends on androgens (T, dihydrotestosterone (DHT)), disturbances in the balance of this endocrine system by either endogenous or exogenous factors lead to male reproductive tract malformation.	strong
Male reproductive tract malformations	Androgens regulate masculinization of the external genitalia. Therefore any defects in androgen biosynthesis, metabolism or action during foetal development can reproductive tract malformation.	strong
Fertility, impaired	Impaired fertility is the endpoint of reproductive toxicity	strong

Weight of Evidence Summary

KERs	Biological plausibility	Level of confidence	Empirical Support			Level of confidence	Inconsistencies/Uncertainties
			Dose-response	Temporality	Incidence		
PPAR alpha, Activation => ***Translator protein (TSPO), Decrease***	There is functional relationship between PPARα activation and reduction in TSPO levels.	Very Weak	• KEs occur at similar dose levels	• occurrence of the key events at similar dose and time point • Support for solid temporal relationship is lacking		Very Weak	Some conflicting data
PPAR alpha, Activation => ***Steroidogenic acute regulatory protein (StAR), decrease***	There is functional relationship between PPARα activation and reduction in StAR levels.	Weak	• KEs occur at similar dose levels	• Support for solid temporal relationship is lacking.		Weak	Some conflicting data
Steroidogenic acute regulatory protein (StAR), decrease and Translator protein (TSPO), Decrease => ***cholesterol transport in mitochondria, reduction***	Changes in cholesterol transport proteins can generally be assumed to directly impact levels of cholesterol transport.	Moderate	• KEs occur at similar dose levels	• Support for solid temporal relationship is lacking.		Moderate	Some conflicting data
cholesterol transport in mitochondria, reduction => ***testosterone synthesis, reduction***	Decreasing the amount of cholesterol inside the mitochondria (e. g by decreasing the expression of enzymes like StAR or TSOP) will result in a diminished amount of substrate for hormone (testosterone) synthesis.	Moderate	• KEs occur at similar dose levels	• occurrence of the key events at similar dose and time point • Support for solid temporal relationship is lacking.		Moderate	Some conflicting data
testosterone, reduction => ***Male reproductive tract malformations***	Reduction in testosterone (T) levels produced in the Leydig cell subsequently lowers the availability of its metabolite; Dihydrotestosterone (DHT).that regulates masculinization of external genitalia. Therefore any defects in androgen biosynthesis, metabolism or action during development can cause male reproductive tract malformation.	Strong	• KEs occur at similar dose levels	• occurrence of the key events at similar dose and time point • Support for solid temporal relationship is lacking.		Strong	No conflicting data

Male reproductive tract malformations => ***Fertility, impaired***	Male reproductive tract malformations (congenital malformation of male genitalia) comprise any physical abnormality of the male internal or external genitalia present at birth, which may impair on fertility later in life	Moderate	• KEs occur at similar dose levels	• occurrence of the key events at similar dose and time point Support for solid temporal relationship is lacking.		Moderate	No conflicting data

Table 1 Weight of Evidence Summary Table. The underlying questions for the content of the table: Dose-response Does the empirical evidence support that a change in KEup leads to an appropriate change in KEdown?; Does KEup occur at lower doses and earlier time points than KE down and is the incidence of KEup > than that for KEdown?: Incidence Is there higher incidence of KEup than of KEdown?; Inconsistencies/Uncertainties: Are there inconsistencies in empirical support across taxa, species and stressors that don't align with expected pattern for hypothesized AOP? n.a not applicable

Quantitative Consideration

This AOP is qualitatively described; however it contains also data that may be used for further development of quantitative description.

Considerations for Potential Applications of the AOP (optional)

1. The AOP describes a pathway which allows for the detection of sex steroid--related endocrine disrupting modes of action, with focus on the identification of substances which affect the reproductive system. In the current form the pathway lays a strong basis for linking endocrine mode of action with an apical endpoint, a prerequisite requirement for identification of endocrine disrupting chemicals (EDC).

EDCs require specific evaluation under REACH (1907/2006, Registration, Evaluation, Authorisation and Restriction of Chemicals (EU, 2006)), the revised European plant protection product regulation 1107/2009 (EU, 2009) and use of biocidal products 528/2012 EC (EU, 2012).Amongst other agencies the US EPA is also giving particular attention to EDCs (EPA, 1998).

2. This AOP structurally represents current knowledge of the pathway from PPARα activation to impaired fertility that may provide a basis for development (and interpretation) of strategies for Integrated Approaches to Testing Assessment (IATA) to identify similar substances that may operate via the same pathway related tosex steroids disruptionand effects on reproductive tract and fertility. This AOP forms the starting point on an AOP network mapping to modes of action for endocrine disruption.

3. The AOP could inform the development of quantitative structure activity relationships, read-across models, and/or systems biology models to prioritize chemicals for further testing.

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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.

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List of Key Events in the AOP

Event: 266: Decrease, Steroidogenic acute regulatory protein (STAR) (https://aopwiki.org/events/266)

Short Name: Decrease, Steroidogenic acute regulatory protein (STAR)

Key Event Component

Process	Object	Action
gene expression	STAR	decreased

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)	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	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
human	Homo sapiens	Moderate	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)

StAR has been cloned from many species, and is highly conserved among mammals, birds, amphibians and fish (Bauer et al. 2000).

Key Event Description

Biological state

Steroidogenic acute regulatory protein (StAR) functions as a cholesterol transfer protein and acts directly on lipids of the outer mitochondrial membrane to promote cholesterol translocation (Stocco 2001). Reduction of the protein impacts on the amount of substrate available for steroidogenesis.

Biological compartments

StAR is expressed principally in steroidogenic tissues (Bauer et al. 2000).

General role in biology

StAR is required for cholesterol shuttling across the mitochondrial membrane and appears to regulate acute steroid production (Clark and Stocco, 1997). Transcriptional or translational inhibition of StAR expression results in a dramatic decrease in steroid biosynthesis, whereas ~10–15% of steroid synthesis appears to be mediated through StAR-independent mechanisms (Manna et al. 2001) (Clark and Stocco, 1997). In contrast, chronically regulated steroid production appears to be largely mediated by increased transcription of steroidogenic enzymes (Hum and Miller 1993).

How it is Measured or Detected

The StAR expression can be measured by RT-PCR (mRNA) and on the protein level (western blot). The StAR expression as well as other steroidogenic proteins 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).

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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)

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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.

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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

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Event: 289: Decrease, Translocator protein (TSPO) (https://aopwiki.org/events/289)

Short Name: Decrease, Translocator protein (TSPO)

Key Event Component

Process	Object	Action
gene expression	translocator protein	decreased

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)	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
Homo sapiens	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)
Mus musculus	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)

TSPO is a protein that shows high DNA sequence conservation from bacteria to mammals. It is expressed ubiquitously, but most abundant in steroidogenic cells (Yeliseev, Krueger, and Kaplan 1997).

Key Event Description

Biological state

Translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor (PBR), is a mitochondrial outer membrane protein implicated in cholesterol import to the inner mitochondrial membrane (Besman et al. 1989).

Biological compartments

The TSPO is present in virtually all mammalian peripheral tissues (Zisterer and Williams 1997), however highly prominent TSPO protein expression has been identified in steroidogenic tissues (R. R. Anholt et al. 1985), (Wang, Fan, and Papadopoulos 2012). The presence of TSOP has been confirmed in Leydig and Sertoli cells (Morohaku, Phuong, and Selvaraj 2013), granulosa cells (Amsterdam and Suh 1991) and to a lesser extent in thecal cells (Morohaku, Phuong, and Selvaraj 2013). In subcellular fractions, binding sites for the TSOP have been identified to be present in the outer mitochondrial membrane (OMM) (R. R. Anholt et al. 1985), (R. Anholt et al. 1986). Transcriptional regulation of TSPO genes has been examined and recently reviewed (Morohaku, Phuong, and Selvaraj 2013).

General role in biology: regulation of lipid transport

TSPO mediates the delivery of the substrate cholesterol to the inner mitochondrial side chain cleavage enzyme P450scc (Besman et al. 1989). TSPO ligands stimulate steroidogenesis and induce cholesterol movement from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) (Besman et al. 1989).

How it is Measured or Detected

TSPO levels can be assayed by standard methods for assessment of gene expression levels like qPCR or direct protein levels by Western blot.

The level of TSPO as well as other steroidogenic protein 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).

Uncertainties and Inconsistencies

This information needs to be moved to a key event relationship page.

TSPO -knockout mice have shown embryonic lethality (Lacapère and Papadopoulos 2003); in contrast recent findings have shown no effect on viability of foetuses (Tu et al. 2014). Aberrant TSPO levels have been linked to multiple diseases, including cancer, endocrine disorders, brain injury, neurodegeneration, ischemia-reperfusion injury and inflammatory diseases (Wang, Fan, and Papadopoulos 2012). However, recent studies have shown opposite results. Peripheral benzodiazepine receptor/translocator

protein global knock-out mice are viable and show no effects on steroid hormone biosynthesis (Tu et al. 2014), (Morohaku et al. 2014). As stated in a recent review "At this point in time, a functional designation for TSPO is still actively being sought" (Selvaraj, Stocco, and Tu 2015).

References

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Lacapère, J.J. & Papadopoulos, V., 2003. Peripheral-type benzodiazepine receptor: structure and function of a cholesterol-binding protein in steroid and bile acid biosynthesis. Steroids, 68(7-8), pp.569–85.

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Tu, L.N. et al., 2014. Peripheral benzodiazepine receptor/translocator protein global knock-out mice are viable with no effects on steroid hormone biosynthesis. The Journal of biological chemistry, 289(40), pp.27444–54.

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Yeliseev, A.A., Krueger, K.E. & Kaplan, S., 1997. A mammalian mitochondrial drug receptor functions as a bacterial “oxygen” sensor. Proceedings of the National Academy of Sciences of the United States of America, 94(10), pp.5101–6. Zisterer, D.M. & Williams, D.C., 1997. Peripheral-type benzodiazepine receptors. General pharmacology, 29(3), pp.305–14.

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.

Event: 348: Malformation, Male reproductive tract (https://aopwiki.org/events/348)

Short Name: Malformation, Male reproductive tract

Key Event Component

Process	Object	Action
	male reproductive organ	morphological change

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)	AdverseOutcome

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
male reproductive system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	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)

Hypospadias

Rodents (Gray et al. 2001) Human (Manson and Carr 2003) Wildlife species (Hayes et al. 2002)

AGD Across numerous species, including humans, AGD is longer in males compared to females; for review see (Barrett et al. 2014).

Key Event Description

Biological state

Male reproductive tract malformations (congenital malformation of male genitalia) comprise any physical abnormality of the male internal or external genitalia present at birth. Some result from excessive or deficient androgen effect, others result from teratogenic effects, or are associated with anomalies of other parts of the body in a recognizable pattern (i.e., a syndrome). The cause of many of these birth defects is unknown.

Hypospadias is a defect of the urogenital system, a malformation in which the urethra opens on the underside of the penis instead of the tip. It results from an incomplete closure of the urethral folds, leaving a split on the penis (Kalfa, Philibert, and Sultan 2009). When the urethra opens to the glans or corona of the penis, it is called distal, whereas opening to the shaft or penoscrotal area defines hypospadias as proximal. Androgens regulate the masculinization of external genitalia. Therefore any defects in androgen biosynthesis, metabolism or action during foetal development can cause hypospadias. Gene defects causing disorders of testicular differentiation, conversion of testosterone to dihydrotestosterone or mutations in the androgen receptor can also result in hypospadias (Kalfa et al. 2008). In about 20% of patients with isolated hypospadias there are signs of endocrine abnormalities by the time of diagnosis (Rey et al. 2005). The majority of hypospadias are believed to have a multifactorial etiology, although a small percentage do result from single gene mutations (Baskin, Himes, and Colborn 2001). The only treatment of hypospadias is surgery, thus, prevention is imperative.

Biological compartments: reproductive system

How it is Measured or Detected

Malformations are detected by macroscopically for any structural abnormality or pathological change. The Congenital malformation of the genitalia is a medical term referring to a broad category of conditions that for humans is classified by International Classification of Diseases (ICD) in chapter "Congenital malformations of genital organs" (Q50-Q56) e.g.Q54 Hypospadias, Q53 Undescended testicle. Hypospadias is usually diagnosed during the routine examination after birth. The hypospadias belongs to the category of "Congenital malformation of the genitalia" - a medical term referring to a broad category of conditions as classified in the International Classification of Diseases (ICD) in chapter "Congenital malformations of genital organs" (Q50-Q56) e.g. Q54 Hypospadias.

The anogenital distance (AGD) is a sexual dimorphism that results from the sex difference in foetal androgen (DHT) levels (Rhees et al., 1997). The AGD, the distance from the anus to the genitals, is widely used as biomarker of prenatal androgen exposure during a reproductive programming window (Wolf et al. 1999), (McIntyre, Barlow, and Foster 2001), (Macleod et al. 2010). The AGD is a marker of perineal growth and caudal migration of the genital tubercle. It is androgen-dependent in male rodents (Bowman et al. 2003). Measurement of AGD has also been proposed as a quantitative biomarker of foetal endocrine disruptor exposure in humans (Arbuckle et al. 2008), (Dean and Sharpe 2013). A longer (more “masculine”) AGD is typically associated with favourable health outcomes, while a shorter AGD is associated with adverse health outcomes. The AGD in males is approximately double that of females. Less is known about clinical correlates of AGD in females, although one study found that in women a longer AGD was associated with increased odds of multifollicular ovaries (Mendiola et al. 2012). The AGD is reflecting the prenatal hormonal milieu and in addition a biomarker for the risk of reproductive health problems linked to that early hormonal environment (Barrett et al. 2014). In animal studies, AGD measured from the genital tubercle to the anus is a sensitive marker of in utero exposure to androgens and anti-androgens, and is used extensively in animal reproductive toxicology studies (McIntyre, Barlow, and Foster 2001). AGD of each pup should be measured on at least one occasion from pre natal day postnatal day (PND) 0 through PND 4. Pup body weight should be collected on the day the AGD is measured and the AGD should be normalized to a measure of pup size, preferably the cube root of body weight (12). AGD is influenced by the body weight of the animal and therefore, this should be taken into account when evaluating the data (Gallavan et al, 1999). Body weight as a covariable may also be used (Howdeshell et al. 2007). Decreased AGD in male rats is a hallmark of exposure to antiandrogenic substances (Noriega et al, 2009; Christiansen et al, 2010). A statistically significant change in AGD that cannot be explained by the size of the animal indicates an adverse effect of exposure and should be considered in setting the NOAEL (OECD, 2008).

The extended one-generation in vivo reproductive toxicity study OECD TG 443 [1] (http://www.oecd-ilibrary.org/environment/test-no-443-extended-one-generation-reproductive-toxicity-study_9789264122550-en)is used to investigate adverse effects of chemical substances on fertility and developmental toxicity in the rat, in which AGD is measured.

Regulatory Significance of the AO

In regulatory hazard identification and risk assessment of chemicals malformations of male genitalia are considered as a chemically induced adverse outcome that is used for risk assessment and management purposes. The prenatal developmental toxicity study (TG 414) is the method for examining embryo-foetal toxicity as a consequence of exposure during pregnancy. Parental and offspring growth, development and viability are the relevant endpoints in generation studies (OECD TG 415/416/443). These guidelines are implemented in a number of occasions where the reproductive /developmental toxicity have to be assessed in order to comply with relevant EU regulations.

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.

AGD is a reproductive endpoint, assessment of AGD is mandatory in OECD TG 443, 415/416 (OECD 2012).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 436: Decrease, Steroidogenic acute regulatory protein (STAR) leads to Reduction, Cholesterol transport in mitochondria (https://aopwiki.org/relationships/436)

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**	

Evidence Supporting Applicability of this Relationship

Rat see Table 1.

Key Event Relationship Description

Steroidogenic acute regulatory protein (StAR) mediates the cholesterol transport from the outer to the inner mitochondrial membrane, where it undergoes side chain cleavage by cytochrome P-450 enzyme (P450scc) that yields the steroid precursor, pregnenolone (Besman et al. 1989). The cholesterol transfer within the mitochondria is the rate-limiting step in the production of steroid hormones. Therefore reduced amount/activity of the StAR impairs the cholesterol delivery that is necessary for the hormone biosynthesis.

Evidence Supporting this KER

Biological Plausibility
The first step in steroidogenesis takes place within mitochondria. StAR facilitates the movement of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) for steroidogenesis [reviewed in (Miller and Auchus 2011)]. It is primarily present in steroid-producing cells, including theca cells and luteal cells in the ovary, Leydig cells in the testis and cells in the adrenal cortex.

Empirical Evidence
Down-regulation of StAR and impaired steroidogenesis was reported upon exposure to phthalates i.a. (Barlow et al. 2003), (Borch et al. 2006), for details see Table 1.

			KE: StAR, decrease	**KE: Cholesterol transport, decrease**		
Compound	Species	Effect level			Details	References
Phthalate (DBP)	rat	LOEL=500 mg/kg/day	mRNA StAR decrease (by ~34%)	reduced Leydig cell lipid content		(Barlow et al. 2003)
Phthalate (DBP)	rat	LOEL=500 mg/kg/day (GD12-19)		decrease uptake of cholesterol Leydig cell mitochondria	decreased testosterone, decreased expression of scavenger receptor B1, P450(SCC), steroidogenic acute regulatory protein, and cytochrome p450c17	(Thompson, Ross, and Gaido 2004)
Phthalate (DBP)	rat	LOEL=500 mg/kg	mRNA and protein StAR decrease		1 dose, Time course analysis (0,5,1,2,3,6,12,18, 24h killed at GD), decreased testosterone in foetal testis	(Thompson et al. 2005)
Phthalate (DEHP)	rat	LOEL=300 mg/ kg/day	mRNA StAR decrease		dose-dependently reduced StAR, TSOP mRNA (GD 21 testes), also on protein levels in Leydig cells	(Borch et al. 2006)
Phthalate (DBP)	rat	LOEL=500 mg/kg/day, (GD12 to 21)	mRNA StAR decrease		Testes examined GD 16, 19, and 21, cytochrome P450 side chain cleavage, cytochrome P450c17, decrease. Testicular testosterone and androstenedione decreased (GD 19 and 21)	(Shultz 2001)
Phthalate (MEHP)	rat	LOEC=250 µM	protein StAR decrease (immature and adult Leydig cells)	cholesterol transport, decrease (into the mitochondria of immature and adult Leydig cells	decreased testosterone by approximately 60%, in vitro (immature and adult Leydig cells)	(Svechnikov, Svechnikova, and Söder 2008)
Phthalate (DBP)	rat	LOEL=500 mg/kg/day	mRNA StAR decrease		GD 12 -20, examinations on GD20	(Johnson et al. 2011)

Table 1 Summary table of empirical support for this KER. LOEC-lowest effect concentration, LOEL- lowest observed effect level, Dibutyl phthalate (DBP), Di-2-ethylhexyl phthalate (DEHP), mono(2-ethylhexyl) phthalate (MEHP).

Uncertainties and Inconsistencies

Some steroidogenesis is independent of StAR; when nonsteroidogenic cells are transfected with the P450scc system, they convert cholesterol to pregnenolone at about 14% of the StAR-induced rate (Lin et al. 1995). The mechanism of StAR-independent steroidogenesis is unclear (Miller and Auchus 2011). Johnson et al proposed the involvment of sterol regulatory element–binding protein (SREBP) in phthalate mediated disruption of steroidogenesis. Their study showed lipid metabolism pathways transcriptionally regulated by SREBP were inhibited in the rat but induced in the mouse, and this differential species response corresponded with repression of the steroidogenic pathway. In rats exposed to 100 or 500 mg/kg DBP from gestational days (GD) 16 to 20, a correlation was observed between GD20 testis steroidogenic inhibition and reductions of testis cholesterol synthesis endpoints including testis total cholesterol levels (Johnson et al. 2011).

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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).

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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).

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Relationship: 437: Decrease, Translocator protein (TSPO) leads to Reduction, Cholesterol transport in mitochondria (https://aopwiki.org/relationships/437)

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**	**Low**	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

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

Rat (Papadopoulos et al., 1997)

Key Event Relationship Description

Translocator Protein (TSPO) mediates the first step of cholesterol transport to the inner mitochondrial membrane cytochrome P-450 side chain cleavage enzyme (P450scc) (Besman et al. 1989). TSPO ligands stimulate steroidogenesis and induce cholesterol movement from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) (Besman et al. 1989). Therefore reduced amount/activity of the TSPO impairs the cholesterol delivery that is necessary for the hormone biosynthesis.

Evidence Supporting this KER

Biological Plausibility

The TSPO was first identified as a peripheral tissue diazepam binding site [known as peripheral-type benzodiazepine receptor (PBR)] and since then it has been implicated in many cellular processes. Amongst these are steroid biosynthesis, protein import, heme biosynthesis, immunomodulation, cellular respiration and oxidative processes. The TSPO is present in virtually all mammalian peripheral tissues (Zisterer and Williams 1997), however highly prominent TSPO protein expression has been identified in steroidogenic tissues (R. R. Anholt et al. 1985),(Wang, Fan, and Papadopoulos 2012). The presence of TSPO was confirmed in Leydig and Sertoli cells (Morohaku, Phuong, and Selvaraj 2013), granulosa cells (Amsterdam and Suh 1991) and to lesser extent in thecal cells (Morohaku, Phuong, and Selvaraj 2013). In subcellular fractions, binding sites for the TSPO were identified to be present in the OMM (R. R. Anholt et al. 1985), (R. Anholt et al. 1986).

Empirical Evidence

The decreased TSPO protein levels leads to decreased cholesterol transport into Leydig cells (Gazouli 2002), (Borch et al. 2006). Moreover, Thompson et al., observed decreased uptake of cholesterol in Leydig cell mitochondria upon exposure to phthalates (Thompson, Ross, and Gaido 2004).

Uncertainties and Inconsistencies

Targeted disruption of TSPO in rat Leydig R2C cells reduced steroidogenesis (Papadopoulos et al. 1997). However, recent experiments with TSPO knockdown in steroidogenic cells was not shown to affect steroid hormone biosynthesis (Tu et al. 2014) as well as in a specific deletion of TSPO in steroidogenic Leydig cells did not impair their synthesis of testosterone (Morohaku et al. 2014). As stated in the recent review "At this point in time, a functional designation for TSPO is still actively being sought" (Selvaraj, Stocco, and Tu 2015).

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Relationship: 405: Malformation, Male reproductive tract leads to impaired, Fertility (https://aopwiki.org/relationships/405)

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**	

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.	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10095)

Human and Rat see "Empirical Support for Linkage"

Key Event Relationship Description

Impairment in the normal development of the male reproductive tract (e.g. genital abnormality and/or cryptorchidism) can impact on fertility later in life.

Evidence Supporting this KER

Biological Plausibility
Hypospadias next to cryptorchidism belongs to the most common male reproductive disorders that manifest at birth and may have a common origin in foetal life (Skakkebaek, Rajpert-De Meyts, and Main 2001) and are associated with decreased fertility (Thorup et al. 2010).

Empirical Evidence
Human

Asklund et al that semen quality was reduced in men with hypospadias and additional genital disorders, predominately cryptorchidism (Asklund et al. 2010). In another study by Bracka, 25% of 41 hypospadias patients including 26 patients also with cryptorchidism had a lower sperm density (Bracka 1989). Men with a history of cryptorchidism have an increased risk of infertility (Thorup et al. 2010). Eisenberg et al. found shorter AGD among infertile men as compared with fertile men (Eisenberg et al. 2011).

Rat

In rodents in utero exposure to agents known to disrupt androgen mediated pathways corrupts normal male genital development with a decrease in genital length (ie phallus length, AGD) and impaired testosterone and sperm production (Macleod et al. 2010), (Cowin et al. 2010), including exposure to phthalates (NTP 2005).

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List of Non Adjacent Key Event Relationships

Relationship: 369: Activation, PPARα leads to Decrease, Steroidogenic acute regulatory protein (STAR) (https://aopwiki.org/relationships/369)

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)	**non-adjacent**	**Moderate**	

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)
mouse	Mus musculus	Moderate	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10090)
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

The direct link of PPARα in regulation of the cholesterol transport in mitochondria and hormone synthesis derives from studies demonstrating that PPARα may act as indirect transrepressor of the key steroidogenic factor-1 (SF-1) (S. Plummer et al. 2007), (S. M. Plummer et al. 2013). SF-1 is a transcription factor essential for expression of genes involved in steroidogenesis (including Steroidogenic acute regulatory protein (StAR)).

Evidence Supporting this KER

Biological Plausibility
The PPARα is expressed in foetal rat Leydig cells (Boberg et al. 2008), (S. M. Plummer et al. 2013) and in adult rat Leydig cells (Schultz et al. 1999). Recent studies have shown that foetal testes contained PPARα protein–binding peaks in CYP11a, StAR, and CYP17a regulatory regions (S. M. Plummer et al. 2013). Binding of PPARα to promoter of steroidogenic gene occurs at binding sites different from those of SF-1, indicating that PPARα may be an indirect repressor of SF1 binding. Moreover, it is possible that PPARα could act via sequestration of the shared coactivator CBP (S. M. Plummer et al. 2013). PPARα and SF-1 share a common coactivator, CREB-binding protein (CBP), which is present in limited concentrations (McCampbell 2000). Binding of CBP to PPARα could therefore starve SF-1 from a cofactor essential for its transactivation functions. SF-1 controls transcription of the StAR gene (Sugawara et al. 1996). Steroidogenic acute regulatory (StAR) protein plays a critical role in the movement of cholesterol from the outer to the inner mitochondrial membrane (Stocco 2001). Hence, it seems likely that the ability of PPARα to interfere with SF-1 binding/transactivation caused by exposure to chemicals (e.g. phthalates) could affect the StAR expression and the cholesterol transport in mitochondria.

Empirical Evidence
PPARα agonists can suppress Leydig cell steroidogenesis (Gazouli 2002), and downregulate steroidogenic genes including StAR (Borch et al. 2006), (Lehmann et al. 2004), (Liu et al. 2005), for details see Table 1. Moreover, PPARα agonists, which do not directly transrepress the StAR promoter, have been found to downregulate the expression of this gene in steroidogenic tissues (in mice ovaries) (Toda et al. 2003).

Compound	species	KE: PPARα, Activation	KE: StAR, Decrease

Phthalate (MBzP)	human	EC50 = 30 µM human (Hurst and Waxman 2003)	n.a.
Phthalate (DBP)	rodent	EC50 = 21 µM MBzP EC50 = 63 µM (Hurst and Waxman 2003) MBuP	LOEC=50 mg/kg/day (Borch et al. 2006) (Lehmann et al. 2004), (Shultz 2001)
Phthalate (MEHP)	rodent	LOEC=30 µM (Bility et al. 2004)	LOEC=300 mg/kg/day (Borch et al. 2006)
Phthalate (MEHP)	human	Ki=15 µM (Lapinskas et al. 2005) EC50 = 3.2 µM (Hurst and Waxman 2003)	n.a.
Phthalate (DiBP)	rat	LOEC=600 mg/kg/day, decrease of PPARα mRNA, at GD 19 and 21 in testes of foetuses (Boberg et al. 2008)	LOEC=600 mg/kg/day, decrease of StAR mRNA at GD 19 and 21 in testes of foetuses (Boberg et al. 2008)
Econazole	rodent	AC50=0.0729 µM (ToxCastTM Data)	LOEC=25 µM, mouse MA-10 Leydig tumor cell line, Decrease in StAR activity and/or expression (Walsh, Kuratko, and Stocco 2000)
Perfluorooctanoate (PFOA)	mice	3T3-L1 cells transfected with human, mouse and rat PPARα (Vanden Heuvel et al. 2006)	LOEC= 5.0 mg/kg/day mRNA StAR decrease in the testis of wild-type mice, LOEC=1.0 mg/kg/day mRNA StAR decrease in testis of *PPARα-humanized* mice (not in the PPARα –null mice) (Li et al. 2011)
Fenofibrate	mice	PPAR agonist Increase PPARα protein (Toda et al. 2003).	Decrease of StAR (protein level, in mice ovaries) (Toda et al. 2003)

Table 1. Summary table for empirical support of KER. ED50 - half maximal effective concentration, LOEC-lowest observed effect concentration, Bis(2-ethylhexyl) phthalate (DEHP), Dibutyl phthalate (DBP), diisobutyl phthalate (DiBP), mono-sec-butyl phthalate (MBuP), n.a - not available.

Uncertainties and Inconsistencies
Uncertainties

PPARα was also shown to regulate Translator protein (TSPO), which is a mitochondrial outer membrane protein implicated in cholesterol import to the inner mitochondrial (for details see Relationship:370 (https://aopwiki.org/wiki/index.php/Relationship:370)). Moreover, there is evidence that activated PPARα regulates the expression of enzymes involved in steroid metabolism (17β-hydroxysteroid dehydrogenase IV, 11β-hydroxysteroid dehydrogenase I, and 3β-hydroxysteroid dehydrogenase V (Hermanowski-Vosatka et al. 2000), (Corton et al. 1996), (Wong et al. 2002)).

Inconsistencies In utero rat exposure to the PPARα agonist Wy-14,643 did not reduce fetal testis steroidogenic gene expression or testosterone production (Hannas et al. 2012).

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Relationship: 370: Activation, PPARα leads to Decrease, Translocator protein (TSPO) (https://aopwiki.org/relationships/370)

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)	**non-adjacent**	**Low**	

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)
mice	Mus sp.	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10095)
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

Activation of PPARα leads to decreased expression of cholesterol transport (TSPO) gene in steroidogenic cells (e.g. Leydig cell) and as a consequence the amount of cholesterol transported into mitochondria decreases (impact on steroid production).

Evidence Supporting this KER

Biological Plausibility

PPARs are nuclear receptors that among many other functions regulate genes involved in cholesterol uptake and transport (Xie, Yang, and DePierre 2002), (Gazouli 2002), (Campioli et al. 2011). The indirect link of PPAR receptors in regulation of the cholesterol transport in mitochondria derives from studies demonstrating PPARα dependent control of TSOP (Gazouli 2002), (Campioli et al. 2011). PPARα is present in steroidogenic cells e.g. of the testes during its development as well as in adult testes

(Schultz et al. 1999), (Boberg et al. 2008) and modulation of its activity has been shown to impact on TSOP transcriptional activity (Gazouli 2002). The exact mechanisms of this relationship are not known.

Empirical Evidence

Gazouli et al showed that PPAR activators (Bezafibrate , MEHP) inhibited the transfer or loading of cholesterol to the inner mitochondrial membrane in MA-10 mouse Leydig tumor cells and decreased levels of TSOP protein. Additionally, in R2C (Leydig tumor cell line) inhibited the formation of progesterone. The levels of the TSPO protein were decreased in testes of adult mice exposed to and DEHP (Gazouli 2002). Moreover, the finding that benzafibrate and phthalates inhibit the hormone-induced and constitutively sustained steroidogenesis with similar IC50 values indicates that these compounds act on a common regulatory component of the steroidogenic pathway (Gazouli 2002). In in vivo studies with rats exposed to DEHP the levels of TSOP (mRNA) were decreased dose-dependently in foetal testes (GD 21 testes), also on protein levels in Leydig cells (Borch et al. 2006). For details see Table1.

Compound	KE: PPARα, Activation	KE: TSPO, Decrease	species	Details	References
Phthalate (DBP)	EC50=30μM	n.a.	human	Monobenzyl phthalate (MBzP) metabolite of DBP	(Hurst and Waxman 2003)
Phthalate (DBP)	EC50=21μM * EC50=63μM**	LOEC=100 μM	rodent	*MBzP **mono-*sec*-butyl phthalate (MBuP) metabolite of DBP	(Hurst and Waxman 2003),(Gazouli 2002)
Phthalate (DEHP)	LOEC=30μM	LOEC=300 mg/kg /d	rodent	MEHP metabolite of DEHP	(Bility et al. 2004), (Gazouli 2002), (Borch et al. 2006)
Phthalate (DEHP)	Ki=15μM; EC50 = 3.2μM	n.a.	human	MEHP metabolite of DEHP	(Lapinskas et al. 2005), (Hurst and Waxman 2003)
Bezafibrate	EC50=55μM	LOEC=100 μM	rodent	TSPO decreased by 80% in MA-10 Leydig cells	(Willson et al. 2000), (Gazouli 2002)
WY-14,643	EC50=0.00027μM	LOEC=50 mg/kg/d	rodent		(Pinelli et al. 2005), (Gazouli 2002)

Table 1. Summary table of empirical support for this KER. ED50 - half maximal effective dose, LOEC-lowest observed effect concentration, Bis(2-ethylhexyl) phthalate (DEHP), Dibutyl phthalate (DBP), WY-14,643 and Bezafibrate ligands of PPARα, n.a.- not available

Uncertainties and Inconsistencies

The exact mechanisms of this relationship are not known.

Treatment of adult mice with PPARα activator (DEHP or WY-14,643) resulted in reduced levls of circulating testosterone and testis TSPO mRNA, consistent with the in vitro effects (Gazouli 2002). In contrast, liver TSPO mRNA levels have been increased, indicating a tissue-specific regulation of TSOP expression by PPARα activator (Gazouli 2002). In the PPARα-null mice, compared with the wild-type controls, circulating testosterone levels were decreased suggesting a positive constitutive role for PPARα in maintaining Leydig cell steroid formation. Surprisingly, treatment of the PPARα-null mice with PPARα activators (DEHP and WY-14,643) restored testosterone formation and TSPO mRNA returned to normal levels, suggesting PPARα-independent pathways might be involved in the regulation of TSPO genes and steroidogenesis (Gazouli 2002). In support of this hypothesis, an other study demonstrated that part of the toxic effect of phthalate (DEHP) on testis was retained in PPARα-null mice (Ward et al. 1998).

There is some evidence involving additional PPARs in transcriptional regulation of TSPO:

- PPARβ/δ (Campioli et al. 2011);
- PPARγ isoform was also detected in testes (Boberg et al. 2008) and it was reduced by treatment of DEHP in parallel with the reduction of TSPO regulation (Borch et al. 2006).

A genomic study does not support the hypothesis that activation of PPARα/γ pathways is involved in the effects of phthalates on sexual differentiation of the male rat, as Wy-14,643 (PPARα activator) has no effect on testosterone production and the PPARγ isoform has not been detected in testes at gestation day 14-18 (Hannas et al. 2012). Differential patterns of TSPO expression in the foetal rat testis have been observed upon phthalate (DBP) treatment, whereas TSPO mRNA up-regulated protein levels were decreased in Leydig cells (Lehmann et al. 2004).

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Relationship: 608: Reduction, testosterone level leads to Malformation, Male reproductive tract (https://aopwiki.org/relationships/608)

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)	**non-adjacent**	**High**	

Evidence Supporting Applicability of this Relationship

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)
mice	Mus sp.	Low	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=10095)

Hypospadias

Maternal exposure to estrogenic and antiandrogenic endocrine disrupting compounds has been implicated in increased risk of cryptorchidism and hypospadias in human male offspring without statistical significance (Morales-Suárez-Varela et al. 2011).

AGD

Across numerous species, including humans, AGD is longer in males compared to females; for review see (Barrett et al. 2014).

Key Event Relationship Description

Male sexual differentiation in general depends on testosterone (T), dihydrotestosterone (DHT), and the expression of androgen receptors by target cells (Manson and Carr 2003). Disturbances in the balance of this endocrine system by either endogenous or exogenous factors may lead to male reproductive tract, malformations (e.g. hypospadias, cryptorchidism). Reduction in T levels during foetal development subsequently lower levels of its metabolite DHT lead also to impaired growth of the perineum with reduced anogential distance (AGD) (Bowman et al. 2003).

Evidence Supporting this KER

Biological Plausibility
Hypospadias

The role of foetal androgens (T and DHT) is crucial for the development of the male reproductive tract especially during the first trimester of pregnancy. Androgens regulate masculinization of external genitalia. T is necessary for stabilization and differentiation of the Wolffian structures (e.g., the epididymis, vas deferens and seminal vesicles) and also for normal development of the foetal testes; DHT, produced locally from testosterone, is required for normal development of the genital tubercle and urogenital sinus into the external genitalia and prostate (Murashima et al. 2015). Therefore any defects in androgen biosynthesis, metabolism or action during development can cause hypospadias (Rey et al. 2005). The environmental factors with anti-androgenic activity may alter the complex regulation of male sex differentiation during foetal life (Kalfa et al., 2008). Although the cause in most cases is unknown, hypospadias has been associated with aberrant androgen signalling during development (Wolf et al. 1999). The aetiology of this frequent malformation has not been elucidated despite intensive investigation (Kalfa, Philibert, and Sultan 2009). Hypospadias thus appears at the crossroads of genetic, endocrine and environmental mechanisms (Kalfa, Philibert, and Sultan 2009).

Anogential distance (AGD)

The anogenital distance (AGD) is a sexual dimorphism that results from the sex difference in foetal androgen (DHT) levels (Rhees et al., 1997). The AGD is a marker of perineal growth and caudal migration of the genital tubercle. It is androgen-dependent in male rodents (Bowman et al. 2003). During development, androgens stimulate the growth of the perineal region between the sex papilla and the anus, resulting in an increased AGD in male offspring (Bowman et al. 2003). The AGD, is believed to be a biomarker of prenatal androgen exposure in many species, and in humans it has been associated with several adverse reproductive health outcomes in adults. AGD reflects foetal androgen exposure only within a discrete masculinization programming window (MPW), during which development of male reproductive organs is taking place (Wolf et al. 1999), (Macleod et al. 2010).

Cryptorchidism

Undescended testis (UDT), also called cryptorchidism, is the most frequent congenital malformation in males, occurring in 2–5% of full-term male births (Hadziselimovic 2002) (Brucker-Davis et al. 2008). Testosterone and insulin-like peptide 3 (INSL3) are two major Leydig cell hormones that regulate physiological testicular descent during foetal development (Virtanen et al. 2007). Most cases of cryptorchidism remain idiopathic but epidemiological and experimental studies have suggested a role of both genetic and environmental factors. Studies e. g.(Gray et al. 2000) have shown that maternal administration of certain chemicals (phthalate esters) during the critical intrauterine period of sexual differentiation alters development of both androgen- and insl3-dependent tissues. Cryptorchidism is shown to be linked with increased risk of hypofertility and testicular cancer (Fénichel et al. 2015).

Empirical Evidence
Hypospadias

Reduced T production during the male rat development lead to hypospadias (Mylchreest, Cattley, and Foster 1998), (Mylchreest 2000), (Gray et al. 2000), (Parks 2000), (Wilson et al. 2004); this outcome is associated with Leydig cell function.

Anogential distance (AGD)

The decreased AGD has been associated with the perturbation of androgen-mediated development of the reproductive tract in rat males which were exposed to anti-androgens in utero (Wolf et al. 1999), (McIntyre et al. 2000), (McIntyre, Barlow, and Foster 2001). Several studies have demonstrated that exposure to phthalates results in decreased anogenital distance in human males (S. H. Swan et al. 2015), (Bornehag et al. 2015), presumably due to lowered testosterone levels (Suzuki et al. 2012), (Jurewicz and Hanke 2011), (Shanna H Swan et al. 2005), for details see Table 1.

			KE: testosterone, reduction	**KE: AGD, decreased**		
Compound	Species and strain: Doses: duration, [measurement day]	Effect level			Details	References
Phthalates (DEHP)	rat, 0, 10, 30, 100, or 300 mg /kg bw/day : 7-21 GD, [GD 21]	LOEL=-300 mg /kg bw/day	Testicular testosterone levels, reduction, no change plasma testosterone			(Borch et al. 2006)
Phthalates (DEHP)	rat, GD 3- PND 21	LOEL=750 mg /kg bw/day		Anogenital distance decreased	Gestational and lactational	(Moore et al. 2001)
Phthalates (DEHP)	rat	LOEL=750 mg /kg bw/day	reduction in T production, and reduced testicular and whole-body T levels in fetal and neonatal male	Anogenital distance decreased	Exposure from (GD) 14 to postnatal day (PND) 3, AGD reduced by 36% in exposed male	(Parks 2000)
Phthalates (DEHP)	rat	LOEL=15 mg /kg bw/day	Decreased testosterone levels	Effects on Sperm production		(Andrade et al. 2006)
Phthalates (DEHP)	rat	LOEL=5 mg /kg bw/day		chryptorchidism		(Andrade et al. 2006)
Phthalates (DEHP)	rat	LOEL=1.215 mg /kg bw/day	Decreased testosterone levels	Effects on Sperm production		(Andrade et al. 2006)
Phthalates (DnHP)	rat	LOEL=250 mg /kg bw/day		Anogenital distance decreased		(Saillenfait, Gallissot, and Sabaté 2009)
Phthalates (DEHP)	rat	LOEL=300 mg/kg/day		Anogenital distance decreased		(Jarfelt et al. 2005)
Phthalates (DBP)	rat	LOEL=500 mg/kg/day		Anogenital distance decreased	throughout pregnancy until postnatal day 20	(Mylchreest, Cattley, and Foster 1998)
Phthalates dicyclohexyl phthalate (DCHP)	rat	LOEL=6000 ppm		Anogenital distance decreased	F1 and F2 6000 ppm, and decrease of AGD and appearance of areola mammae were observed in the F1 male 6000 ppm and F2 male receiving doses of 1200 ppm or 6000 ppm.	(Hoshino, Iwai, and Okazaki 2005)
Phthalate (DBP)	rat	LOEL=500 mg/kg/day	intratesticular testosterone levels, reduction (by nearly 90%)	Anogenital distance decreased	GD 12 -20, examinations on GD20	(Johnson et al. 2011)
Phthalates (MEHP)	Human			Anogenital index decreased	Urine concentration of phthalates metabolites MEHP associated with reduced AGI, suggestive association of sum of DEHP metabolites with reduced AGI	(Suzuki et al. 2012),
Phthalates (MEP), (MBP), (MBzP), (MiBP)	human			Urinary concentrations of phthalate metabolites inversely related to AGI	134 boys 2-36 months of age	(Shanna H Swan et al. 2005)

Phthalates (MEHP, MBP)	human	MBP= 78.4 ng/mL* in urine; 85.2 ng/mL* in amniotic fluid MEHP =24.9 ng/mL * in urine; 22.8 ng/mL* in amniotic fluid		In girls, decreased AGD in relation to amniotic fluid levels of MBP and MEHP. No associations found in boys	Amniotic fluid and urine concentrations of phthalate metabolites	(Huang et al. 2009)

Table 1 Summary of experimental evidence for the KER. Lowest-Observed-Effect-Level (LOEL), Dibutyl phthalate (DBP), diisobutyl phthalate (DiBP), di-n-hexyl phthalate (DnHP), monobutyl phthalate (MBP); Bis(2-ethylhexyl) phthalate (DEHP) mono-(2-ethylhexyl) phthalate (MEHP); monoethyl phthalate (MEP), monobenzyl phthalate (MBzP), monoisobutyl phthalate (MiBP); anogenital index (AGI)-weight normalised index of AGD median.

Uncertainties and Inconsistencies

Hypospadias

Epidemiological studies have demonstrated an association between foetal estrogen exposure and hypospadias (Klip et al. 2002), (Brouwers et al. 2007). However, the molecular mechanism underlying this association is unknown (Wang and Baskin 2008), (Blaschko, Cunha, and Baskin 2012).

Anogential distance (AGD)

Study by Huang et al did not found associations with the phthalates metabolites in the male AGD, however in females in relation to amniotic fluid levels of MBP and MEHP (Huang et al. 2009).

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