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AOP 6: Antagonist binding to PPARα leading to body-weight loss

Short Title: PPARα antagonism leading to body-weight loss

This document was approved by the Extended Advisory Group on Molecular Screening and Toxicogenomics in June 2018.

The Working Group of the National Coordinators of the Test Guidelines Programme and the Working Party on Hazard Assessment are invited to review and endorse the AOP by 29 March 2019.

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Foreword

This Adverse Outcome Pathway (AOP) on Antagonist binding to PPARα leading to body-weight loss, has been developed under the auspices of the OECD AOP Development Programme, overseen by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), which is an advisory group under the Working Group of the National Coordinators for the Test Guidelines Programme (WNT). The AOP has been reviewed internally by the EAGMST, externally by experts nominated by the WNT, and has been endorsed by the WNT and the Working Party on Hazard Assessment (WPHA) in xxxxx.

Through endorsement of this AOP, the WNT and the WPHA express confidence in the scientific review process that the AOP has undergone and accept the recommendation of the EAGMST that the AOP be disseminated publicly. Endorsement does not necessarily indicate that the AOP is now considered a tool for direct regulatory application.

The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to declassification of this AOP on xxxxxx.

This document is being published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

The outcome of the internal and external reviews are publicly available respectively in the AOP Wiki and the eAOP Portal of the AOP Knowledge Base at the following links: [internal review] [external review].

Table of contents

Adverse Outcome Pathway on Antagonist binding to PPARα leading to body-weight loss

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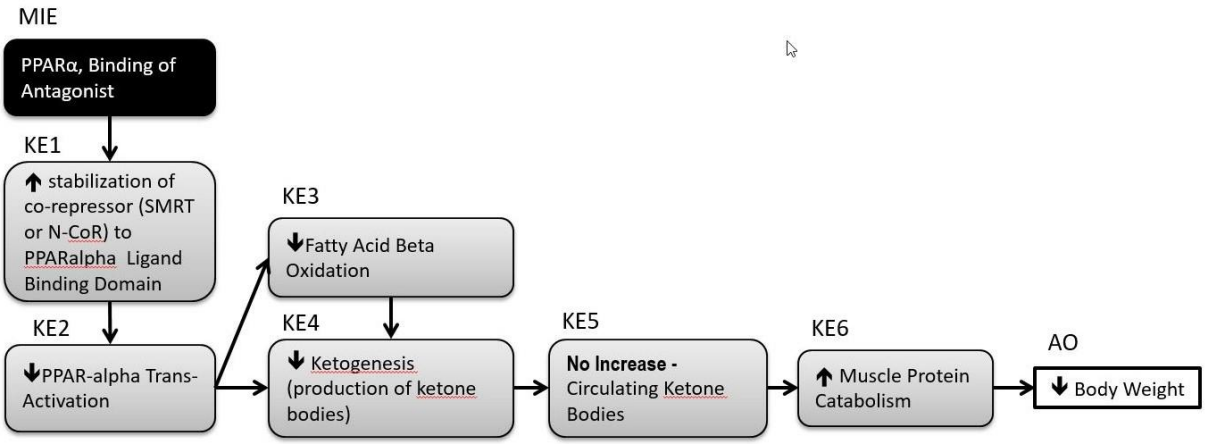
Abstract

The present AOP describes antagonistic chemical binding to the peroxisome proliferator-activated receptor α (PPARα) resulting in preferential binding a co-repressor to the overall PPARα signaling complex causing a chain of events that includes: antagonism of PPARα nuclear signaling, decreased transcriptional expression of PPARα-regulated genes that support energy metabolism, and inhibited metabolic energy production culminating with starvation-like weight loss. The AOP is likely to be synergized during fasting, starvation or malnutrition events. The MIE for this AOP involves antagonistic PPARα binding. The antagonist-binding to the PPARα regulatory complex causes the KE1, stabilization of co-repressor (SMRT or N-CoR) to PPARalpha ligand binding domain suppressing PPARα nuclear signaling (Nagy et al 1999, Xu et al 2002). PPARα is a transcriptional regulator for a variety of genes that facilitate systemic energy homeostasis (Kersten 2014, Evans et al 2004, Desvergne and Wahli 1999). As a result of the MIE and then KE1, the KE2 occurs where PPARalpha transactivation is inhibited for genes involved in the next 2 key events of the AOP: (KE3) decreased fatty acid beta oxidation (Desvergne and Wahili 1999, Kersten 2014, Dreyer et al 1992, Lazarow 1978, Brandt et al 1998; Mascaro et al 1998, Aoyama et al 1998, Gulick et al 1994, Sanderson et al 2008) and (KE4) decreased ketogenesis (Cahil 2006, Kersten et al 2014, Sengupta et al 2010, Desvergne and Wahli 1999). The KE3 results in decreased catabolism of very long chain fatty acids in peroxisomes and reduced catabolism of long, medium and short chain fatty acids in mitochondria reducing acetyl-CoA availability for use in oxidative phosphorylation-based ATP production (Evans et al 2004). KE2 (and also potentially KE3) can drive KE4 resulting in decreased potential to repackage energy substrates as ketone bodies to support systemic energy demands during periods where the systemic energy budget is negative (Badman et al 2007, Potthoff 2009; Muoio et al 2002). The KE5, no change or a decrease in circulating ketone bodies becomes critical during cellular energy deficit conditions, a state where ketogenesis is typically induced to increase circulating ketone bodies providing metabolic fuel to sustain energy homeostasis (Cahill 2006). Physiological studies of the progression of human starvation have demonstrated the critical importance of ketogenesis, especially production of β-hydroxybutyrate, for meeting systemic energy demands by supplementing glucose to sustain the energy requirements of the brain (Cahill 2006, Owen et al 2005). PPARα knock can inhibit ketogenesis from fatty acid substrates in fasted mice reducing β-hydroxybutyrate production causing hypoketonemia (Badman et al 2007, Le May et al 2000, Muoio et al 2002). Sustained negative energy budgets lead to KE6, an increase in muscle protein catabolism, with glutamine and alanine recycled for gluconeogenesis (Felig et al 1970A, Kashiwaya et al 1994). If ketogenesis from fatty acid substrates fails to meet cellular energy needs, gluconeogenesis from alternative substrates becomes necessary including (KE 6) muscle protein catabolism *in situ* supporting local muscle function and releasing glutamine (Marliss et al 1971) and alanine (Felig et al 1970A) for gluconeogenesis in kidney and liver to sustain systemic energy needs (Goodman et al 1966, Kashiwaya et al 1994, Cahill 2006). Finally, the AO of body-weight loss occurs, which within the context of dynamic energy budget theory, decreases energy allocations to organismal maturation and reproduction (Nisbet et al 2000) and has been demonstrated to negatively affect ecological fitness (Martin et al 1987).

Graphical Representation



Summary of the AOP

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

Sequence	Type	Event ID	Title	Short name
1	MIE	998	Binding of antagonist, PPAR alpha	Binding of antagonist, PPAR alpha
2	KE	1000	stabilization, PPAR alpha co-repressor	stabilization, PPAR alpha co-repressor
3	KE	858	Decreased, PPARalpha transactivation of gene expression	Decreased, PPARalpha transactivation of gene expression
4	KE	1528	Fatty Acid Beta Oxidation, Decreased	Fatty Acid Beta Oxidation, Decreased
5	KE	861	Decreased, Ketogenesis (production of ketone bodies)	Decreased, Ketogenesis (production of ketone bodies)
6	KE	862	Not Increased, Circulating Ketone Bodies	Not Increased, Circulating Ketone Bodies
7	KE	863	Increased, Catabolism of Muscle Protein	Increased, Catabolism of Muscle Protein
8	AO	864	Decreased, Body Weight	Decreased, Body Weight

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
stabilization, PPAR alpha co-repressor	adjacent	Decreased, PPARalpha transactivation of gene expression	High	Moderate
Decreased, PPARalpha transactivation of gene expression	adjacent	Fatty Acid Beta Oxidation, Decreased	High	High
Decreased, PPARalpha transactivation of gene expression	adjacent	Decreased, Ketogenesis (production of ketone bodies)	Moderate	Moderate
Fatty Acid Beta Oxidation, Decreased	adjacent	Decreased, Ketogenesis (production of ketone bodies)	Moderate	Low

Decreased, Ketogenesis (production of ketone bodies)	adjacent	Not Increased, Circulating Ketone Bodies	High	Moderate
Increased, Catabolism of Muscle Protein	adjacent	Decreased, Body Weight	High	High
Binding of antagonist, PPAR alpha	non-adjacent	stabilization, PPAR alpha co-repressor	High	Moderate
Not Increased, Circulating Ketone Bodies	non-adjacent	Increased, Catabolism of Muscle Protein	Moderate	Low

Stressors

Name	Evidence
GW6471	High
Nitrotoluenes (hypothesized binding)	Moderate
PPARalpha antagonists	High

GW6471 - Specifically designed molecules such as the PPARα antagonists GW6471 can bind to PPARα selectively recruiting binding of co-repressors to the PPARα nuclear signaling complex (Xu et al 2002).

Nitrotoluenes (hypothesized binding) - Recent observations of PPARalpha antagonism by nitrotoluenes (including: 2,4-dinitrotoluene and 2-amino-4,6-dinitrotoluene) have demonstrated dose-response relationships for PPARalpha nuclear signaling inhibition in *in vitro* investigations which corresponded with dose-responsive decreases in transcriptional expression of genes for which PPARalpha acts as a transcriptional regulator, including various lipid metabolism pathways (Wilbanks et al 2014, Gust et al 2015). There is additional evidence that multiple nitrotoluenes including the base structure, 2,4,6-trinitrotoluene and molecules with various substitutions of the nitro groups around that base structure inhibit PPARalpha nuclear signaling, and have elevated binding affinity to PPARalpha, based on computational docking calculations (Gust, unpublished data).

Overall Assessment of the AOP

The majority of the evidence described in this AOP are derived for either human (mostly in vitro) or mice (in vivo and in vitro) studies. There are recognized differences between mouse and human PPARα signaling and responses from the literature, however, for our specific KEs, the responses among species are relatively well conserved. Therefore, we have reasonable confidence that the AOP provides reliable confidence for human health assessment. The AOP also has the potential to support ecotoxicological assessment if there is reasonable confidence that the KEs are conserved in the species of interest. The risk for this AOP is expected to be exacerbated during fasting, starvation and/or sub-optimal nutrition where interference with PPARα signaling is likely to contribute synergistically toward decreased exercise performance in the short-term and drive body-weight loss in long-term exposures. The molecular responses from the MIE through KE4 are very well characterized in the literature for human and mouse. KE5 and KE6 have fairly strong support from the literature, however the KER between them, especially stemming back to the MIE remains the largest data gap within the AOP. Finally, the connection between KE6 and the AO is intuitive and well established in the literature. Overall, the AOP is biologically plausible with logical order where AO is likely to be exacerbated when nutrition is suboptimal.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI
Colinus virginianus	Colinus virginianus	Moderate	NCBI
Pimephales promelas	Pimephales promelas	Low	NCBI
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI
Homo sapiens	Homo sapiens	High	NCBI

Sex Applicability

Sex	Evidence
Female	High
Male	Moderate

The majority of the evidence described in this AOP are derived for either human (mostly in vitro) or mice (in vivo and in vitro) studies. There are recognized differences between mouse and human PPARα signaling and responses from the literature, however, for our specific KEs, the responses among species are relatively well conserved. Therefore, we have reasonable confidence that the AOP provides reliable confidence for human health assessment. The AOP also has the potential to support ecotoxicological assessment if there is reasonable confidence that the KEs are conserved in the species of interest.

1. Essentiality of the Key Events

Rationale for essentiality calls:

- **MIE: PPARα, Binding of antagonist:** Regarding the present MIE, molecules can bind to the PPARα regulatory complex affecting the binding of co-activators and co-repressors. Specifically designed molecules such as the PPARα antagonists GW6471 can bind to PPARα selectively recruiting binding of co-repressors to the PPARα nuclear signaling complex (Xu et al 2002).
- **Key Event 1: PPAR alpha co-repressor, Increased** - The binding of co-repressors to the PPARα signaling complex suppresses nuclear signaling and thus downstream transcription of PPARα-regulated genes (Liu et al 2008). GW6471 binding to the co-repressor is reversible thus allowing the co-repressor to leave the ligand binding domain of PPARα, restoring normal function (Xu et al 2002).
- **Key Event 2: PPARalpha transactivation of gene expression, Decreased** - As described in a variety of reviews, PPARalpha represents a master regulator of energy metabolism which specifically promotes fatty oxidation for energy production & distribution (Evans et al 2004, Kersten 2014, Lefebvre et al 2006, Desvergne and Wahili 1999). Both PPARalpha knock outs and PPARalpha antagonism decreased transcriptional expression of gene targets involved in peroxisomal fatty acid beta oxidation (Kersten et al 1999, Desvergne and Wahili 1999, Janssen et al 2015), mitochondrial fatty acid beta oxidation (Brandt et al 1998; Mascaro et al 1998, Kersten 2014), and ketogenesis (Sengupta et al 2010, Desvergne and Wahli 1999, Kersten 2014).
- **Key Event 3: Fatty Acid Beta Oxidation, Decreased –** This key event is essential for deriving metabolic energy from fatty acid substrates thus supporting a large component of overall organismal energy demands (Evans et al 2004, Kersten 2014, Desvergne and Wahili 1999). Very long chain fatty acids (>C20) are metabolized in the peroxisome and short, medium and long chain fatty acids (<C20) are catabolized by mitochondrial beta-oxidation. PPARalpha regulates nearly every enzymatic step in the uptake as well as the oxidative breakdown of acyl-CoAs to acetyl-CoA (Kersten 2014). The acetyl-CoA monomers serve as fundamental units for metabolic energy production (ATP) via the citric acid cycle followed by electron-transport chain mediated oxidative phosphorylation (Nelson and Cox, 2000A) as well as serve as the fundamental units for energy storage via gluconeogenesis (Nelson and Cox, 2000B) and lipogenesis (Nelson and Cox, 2000C). PPARalpha knockout studies have demonstrated impaired mitochondrial fatty acid oxidation leading to fatty acid accumulation in the liver (Badmann et al 2007) as well as an inability to meet systemic energy demands (Kersten et al, 1999).
- **Key Event 4: Ketogenesis (production of ketone bodies), decreased** - The liver represents a key organ involved in systemic energy distribution given its ability to synthesize glucose (an ability shared only with the kidney, Gerich et al 2001) as well as its exclusive role in the generation of ketone bodies (Cahill 2006, Sengupta et al 2010, Kersten 2014). This is especially important for the metabolic energy needs of the brain, which can

only use glucose and the ketone body, β-hydroxybutyrate for cellular energy production (Cahill 2006, Owen 2005, Kersten 2014). Therefore, ketogenesis is critical to supporting general systemic energy homeostasis in fasting events (Cahill 2006, Evans et al 2004, Sengupta et al 2010). Interference with ketogenesis, for example by PPARα inhibition, has been demonstrated to inhibit β-hydroxybutyrate production (measured in serum) during fasting events in mice (Le May et al 2000, Badman et al 2007, Potthoff 2009, Sengupta et al 2010) and cause hypoketonemia (Muoio et al 2002). The Badman et al (2007) study indicated that metabolism of fatty acid substrates (measured as liver triglycerides) that would otherwise contribute to β-hydroxybutyrate production was additionally inhibited under PPARα knockout.

- **Key Event 5: Circulating Ketone Bodies, Not Increased -** Physiological studies of the progression of human starvation have identified that the preferred metabolic fuel is glucose in the fed state and progressing through two days of fasting, afterward ketone bodies become increasingly important for meeting energy demands (Cahill 2006, Owen et al 2005). Substrates derived from carbohydrates, fats and protein can contribute to gluconeogenesis (Cahill 2006, Gerich et al 2001) whereas substrates derived from fatty acids are the primary contributors to ketogenesis (Desvergne and Wahli 1999). Cahill (2006) and colleagues have demonstrated the importance of ketone body production, especially β-hydroxybutyrate, for maintaining energy homeostasis during starvation by serving as an alternative substrate to glucose for providing energy to the brain in the starvation state (Cahill 2006). Interference with ketogenesis, for example by PPARα inhibition, has been demonstrated to inhibit β-hydroxybutyrate production (measured in serum) during fasting events in mice (Badman et al 2007, Potthoff 2009). Related to this observation, PPARα-knockout mice reached exhaustion sooner than wild types in an exercise challenge which corresponded with significantly decreased β-hydroxybutyrate in serum indicating hypoketonemia in PPARα-knockout mice versus wild types (Muoio et al 2002). Under normal conditions, activated ketogenesis occurring during fasting events is rapidly deactivated when blood glucose concentrations increase to normal levels and resultant elevated circulating ketone bodies are reduced correspondingly (Cahill 2006).
- **Key Event 6: Catabolism of Muscle Protein, Increased -** After two to three days of fasting in humans, dietary glucose has been long-since expended and contribution to blood glucose from glycogen metabolism is reduced to zero (Cahill 2006). At this point, about two fifths of fatty acid metabolism in the whole body is dedicated to hepatic ketogenesis, largely in support of the energy demands of the brain, however the brain is still significantly supported by glucose derived from gluconeogenesis (Cahill 2006). As fatty acid stores are depleted, gluconeogenesis from other substrates becomes increasingly important including muscle protein catabolism *in situ* for supporting muscle function as well as releasing glutamine (Marliss et al 1971) and alanine (Felig et al 1970A) which can be recycled to glucose by gluconeogenesis in the kidney (Goodman et al 1966, Kashiwaya et al 1994, Cahill 2006). In prolonged starvation events, the catabolism of muscle protein for gluconeogenesis in order to support systemic energy needs results in loss of muscle mass, which contributes to loss of overall body weight. This loss is rapidly reversible upon input of alternative metabolic fuel for example by nutrient assimilation from feeding.
- **Adverse Outcome: Loss of body weight -** If caloric intake is less than caloric use over time, an individual will lose body weight. Dynamic energy budget theory has provided useful insights on how organisms take up, assimilate and then allocate energy to various fundamental biological processes including maintenance, growth, development and reproduction (Nisbet et al 2000). Regarding energy allocation, somatic maintenance must first be met before then growth may occur, followed by maturation and then finally, surplus

energy is dedicated to reproduction (Nisbet et al 2000). The influence of PPARalpha on systemic energy metabolism and energy homeostasis has been broadly established (see reviews by Kersten 2014, Evans et al 2004, Desvergne and Wahli 1999). PPARalpha has been demonstrated to play a critical role in stimulating fatty acid oxidation and ketogenesis during fasting resulting in increased ketone body levels in plasma (Badman et al 2007, Kersten 2014) a response that is eliminated in PPARalpha knockout mice (Badman et al 2007, Sanderson et al 2010). Kersten et al (1999) and Badman et al (2007) demonstrated that PPARalpha-null mice were unable to actively mobilize fatty acid oxidation, and further, Kersten et al (1999) demonstrated that these mice were unable to meet energy demands during fasting and leading to hypoglycemia, hyperlipidemia, hypoketonemia and fatty liver. Observations from toxicological and toxicogenomic research have implicated nitrotoluenes as potential PPAR antagonists in birds (Rawat et al 2010), rats (Deng et al 2011) and mice (Wilbanks et al 2014), an effect that additionally corresponded with weight loss in rats (Wilbanks et al 2014) and body weight loss, loss of muscle mass and emaciation in birds (Quinn et al 2007). These combined results indicate that inhibition of PPARalpha signaling and the resultant decrease in fatty acid oxidation and ketogenesis can detrimentally impair systemic energy budgets leading to starvation-like effects and resultant weight loss. In the absence of PPARalpha knockout, and upon removal of PPARalpha antagonist dosing, normal bioenergetic physiology can potentially be attained.

Weight of Evidence Summary

Biological Plausibility

Binding of molecules to peroxisome proliferator-activated receptor α (PPARα) can cause either agonistic or antagonistic signaling depending on molecular structure (Xu et al 2001, Xu et al 2002). Certain molecules that can bind to the PPARα ligand binding domain have been observed to cause conformational changes that induce increased affinity to co-repressors which decrease PPARα nuclear signaling (Xu et al 2002) representing the MIE for this AOP.

The transcription co-repressors, silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressor (N-CoR) have been observed to compete with transcriptional co-activators for binding to nuclear receptors (including PPARα) thus suppressing basal transcriptional activity (Nagy et al 1999, Xu et al 2002). Regarding the KE1, the binding of co-repressors such as the SMRT and N-CoR to PPARα is reinforced by the MIE, which blocks the AF-2 helix from adopting the active conformation, as demonstrated in x-ray crystallography results presented in Xu et al (2002). Thus, molecules that bind to PPARα that can enhance co-repressor binding act as PPARα antagonists.

Given that PPARα trans-activation induces catabolism of fatty acids, this signaling pathway has been broadly demonstrated to play a key role in energy homeostasis (Kersten 2014, Evans et al 2004, Desvergne and Wahli 1999). In fact, PPARα regulates expression of genes encoding nearly every enzymatic step of fatty acid catabolism including fatty acid uptake into cells, fatty acid activation to acyl-CoAs, the release of cellular energy from fatty acids through the oxidative breakdown of acyl-CoAs to acetyl-CoA, and in starvation conditions, the repackaging of Acetyl-CoA substrates into ketone bodies (Kersten 2014, Desvergne and Wahli 1999, Evans et al 2004, Sengupta et al 2010).

A large body of research demonstrated that PPARα nuclear signaling directly controls transcriptional expression for genes catalyzing peroxisomal beta-oxidation of very long chain fatty acids (>20C), mitochondrial beta-oxidation of short, medium and long chain fatty acids (<20C), and ketogenesis (as reviewed in Kersten 2014, Evans et al 2004, Desvergne and Wahli 1999, Sanderson et al 2010, McMullen et al 2014, Rakhshandehroo et al 2009).

Peroxisomal beta-oxidation reactions shorten very long chain fatty acids from dietary sources releasing acetyl-CoA subunits (a primary metabolic fuel source) and shortened-chain fatty acids that can subsequently be catabolized by mitochondrial fatty acid beta oxidation reaction (as reviewed in Kersten et al 2014 and Desvergne and Wahli 1999). Fatty acids shortened via peroxisomal beta-oxidation as well as fatty acids released from adipose tissue stores can be catabolized in mitochondrial beta-oxidation reactions to acetyl-CoA, NADH and ATP (Aoyama et al 1998). Within the mitochondria, the acetyl-CoA substrates can be used to maximize ATP production through full substrate oxidation via the citric acid cycle followed by oxidative phosphorylation by the electron transport chain (Nelson and Cox 2000A, Desvergne and Wahli 1999). This demonstrates the importance of PPARα signaling for inducing cellular energy release from fatty acids.

Blocking PPARα signaling has been shown to inhibit expression of transcripts / enzymes involved in both peroxisomal and mitochondrial beta-oxidation causing impaired fatty acid catabolism, fatty acid accumulation in the liver and impaired cellular energy state during fasting events (Badman et al 2007, Kersten et al 1999).

During periods of fasting, acetyl-CoA generated during either peroxisomal or mitochondrial beta-oxidation of fatty acids in the liver can each contribute to ketogenesis (Kersten 2014, Sengupta 2010). The liver represents a key organ involved in systemic energy distribution given its ability to synthesize glucose and catalyze the formation of ketone bodies, especially β-hydroxybutyrate, via ketogenesis (Cahil 2006, Kersten 2014). β-hydroxybutyrate is especially important for the metabolic energy needs of the brain which is unable to utilize fatty acids for cellular energy production (Owen 2005, Kersten 2014) as well as supporting general systemic energy homeostasis in fasting events (Cahil 2006, Evans et al 2004).

Not only does PPARα signaling stimulate the release of cellular energy from fatty acids, it also regulates the transcription of enzymes that catalyze the repackaging of that cellular energy to ketone bodies via ketogenesis (Sengupta et al 2010, Desvergne and Wahli 1999). Inhibition of PPARα signaling has been demonstrated to inhibit transcriptional expression of genes that catalyze ketogenesis as well as ketone body production (Badman et al 2007, Potthoff 2009, Sengupta 2010) affecting circulating levels of ketone bodies for systemic use.

Kersten et al (1999) demonstrated that PPARα is induced in fasted mice mobilizing the oxidation of fatty acids for energy production. In that study, PPARα-null mice did not

actively induce fatty acid oxidation or ketogenesis leaving the mice unable to meet energy demands during fasting and leading to hypoglycemia, hyperlipidemia, hypoketonemia and fatty liver. In such energy deficits, muscle protein catabolism is induced where the amino acids glutamine and alanine serve as substrates for gluconeogenesis in the kidney to supplement cellular energy production / distribution (Cahill 2006, Marliss et al 1971, Felig et al 1970A, Goodman et al 1966, Kashiwaya et al 1994).

PPARα knockout resulted in important organism-level responses including quicker onset of exhaustion compared to wild type mice in exercise trials where PPARα-K/Os exhibited hypoketonemia (Muoio et al 2002). Additionally, animals exposed to pollutants (nitrotoluenes) that act as partial PPARα antagonists had decreased exercise endurance (Wilbanks et al 2014), showed body weight loss (Wilbanks et al 2014, Quinn et al 2007) and displayed loss of muscle mass (Quinn et al 2007).

In general, if caloric intake is less than caloric use over time, an individual will lose body weight. This is a basic principle in human dieting as well as an important principle related to individual health and ecological fitness of animal populations.

Dynamic energy budget theory has provided useful insights on how organisms take up, assimilate and then allocate energy to various fundamental biological processes including maintenance, growth, development and reproduction (Nisbet et al 2000). Regarding energy allocation, somatic maintenance must first be met before then growth may occur, followed by maturation and then finally, surplus energy is dedicated to reproduction (Nisbet et al 2000).

As an example of the importance of energy allocation to ecological fitness, a review by Martin et al (1987) demonstrated that energy availability (availability of food) was the predominant limiting factor in reproductive success and survival for both young and parents in a broad life history review for bird species. This is a likely scenario for many organisms.

Concordance of dose-response relationships

Dose-response relationships have been developed for GW6471 and the relative binding of PPARα co-repressors and co-activators to the PPARα nuclear signaling complex where the proportion of co-repressors increases dramatically with increasing GW6471 concentration (Xu et al 2002). Correspondingly, the relative activity of PPARα decreased to zero with increasing GW6471 concentrations (Xu et al 2002). Additionally, recent observations of PPARα antagonism by nitrotoluenes have demonstrated dose-response relationships for PPARα nuclear signaling inhibition in human *in vitro* investigations which corresponded with dose-responsive decreases in transcriptional expression of genes involved in lipid metabolism pathways (Wilbanks et al 2014, Gust et al 2015). These results corresponded with a dose-responsive relationship where increasing nitrotoluene dose caused decreased muscle mass, decreased body weight and increased emaciation in chronic dosing studies (Quinn et al 2007).

Temporal concordance among the key events and adverse effect

Co-repressor binding was observed prior to inhibition of PPARα signaling (Xu et al 2002). PPARα knock out nullifies downstream expression of transcripts for genes involved in peroxisomal beta-oxidation of fatty acids, mitochondrial beta-oxidation of fatty acids, and ketogenesis pathways relative to wild types (Kersten et al 2014). Peroxisomal beta-oxidation of very long chain fatty acids into long chain fatty acids occurs prior to import into mitochondria and progression of mitochondrial beta-oxidation (Lazarow 1978, Kersten 2014). Mitochondrial beta-oxidation of long chain fatty acids occurs prior to generation of ketone bodies via ketogenesis (Sengupta et al 2010, Badman et al 2007). Ketogenesis occurs prior to increases in circulating ketone bodies (Sengupta et al 2010, Badman et al 2007, Cahill 2006). Increases in circulating ketone bodies can be observed prior to loss of muscle mass to muscle-protein catabolism given that this linkage is not directly connected. Muscle protein catabolism derives amino acids that are recycled to glucose via renal gluconeogenesis (Goodman et al 1966, Kashiwaya et al 1994, Cahill 2006). Catabolism of muscle protein occurs prior to body weight loss (Quinn et al 2007).

Consistency

The transcription co-repressors, silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressor (N-CoR) competition with transcriptional co-activators for binding to nuclear receptors (including PPARα) has been observed in humans as well as yeast (Nagy et al 1999) suggest broad taxonomic applicability for this MIE. The evidence of PPARα as a regulator of fatty acid metabolism is well described in the literature (for example, Kersten 2014, Evans 2004, Desvergne and Wahili 1999), and is consistent across many species including human, mouse, rat, Northern bobwhite, fathead minnow and carp (Kersten et al 1999, Kersten 2014, Wintz et al 2006, Gust et al 2015, Deng et al 2011, Wilbanks et al 2014, Xu and Jing, 2012). Inhibition of PPARα via gene knockout or treatment with PPARα antagonist consistently results in deceased fatty acid metabolism with indicators of increased serum triglycerides, fatty livers and steatosis (Kersten 2014, Evans 2004, Desvergne and Wahili 1999, Kersten et al 1999, Wintz et al 2006, Deng et al 2011). Given PPARα's central role in systemic energy metabolism, studies of PPARα antagonism have shown decreased potential for sustaining energy needs of the organism (Kersten et al 1999) leading to decreased exercise performance (Muoio et al 2002, Wilbanks et al 2014) and weight loss (Wilbanks et al 2014, Quinn et al 2007). Research thus far suggest that the PPARα transcriptional regulation pathway as well as the metabolic pathways for which PPARα acts as a regulator indicates that the progression of key events through to the adverse outcome will tend to be evolutionarily conserved for within mammals and likely across animal phyla.

Uncertainties, inconsistencies, and data gaps

A critical data gap regarding this AOP is an absence of studies that have investigated the effects null mutants for ketogenesis on the physiology and individual performance during long term starvation relative to wild type individuals. Additionally, knowledge about feedback mechanisms between ketogenesis vs gluconeogenesis would be beneficial for interpreting systemic energy metabolism. Regarding the antagonistic action of nitrotoluenes on PPARalpha nuclear signaling (Wilbanks et al 2014, Gust et al 2015), receptor-binding assays would be beneficial to determine if this class of chemicals is

binding the SMRT and N-CoR co-repressors, similar to the antagonistic action of GW6471 (Xu et al 2002).

Quantitative Consideration

Given the complex nature of PPARalpha's functioning within a multi-subunit transcription factor regulating the transcriptional expression of a multitude of genes that facilitate lipid metabolism, to our knowledge, the relationship between PPARalpha signaling and individual gene expression has not yet been quantitatively modeled. However, the gene regulatory networks structure is well established (KEGG Pathway, map03320) and numerous empirical observations of the positive relationship between PPARalpha signaling with transcript expression and downstream metabolic pathways (Kersten 2014, Desvergne and Wahli 1999), there is opportunity to develop a quantitative gene signaling model for this system. For peroxisomal and mitochondrial fatty acid beta-oxidation pathways and ketogenesis, a variety of enzyme kinetics information is available for modeling (see reviews by Kersten 2014, Desvergne and Wahli 1999) as well as basic knowledge of the reaction stoichiometry of each metabolic reactions that can contribute to metabolic energy substrates for systemic use. Resultant models should be integrated with the work of Kashiwaya et al (1994) who have developed a detailed quantitative model for the metabolic flux of glucose including the influence of ketone bodies and insulin action on the dynamics of glycolysis versus gluconeogenesis. Dynamic energy budget (DEB) models (Nisbet et al 2000) have strong utility for integrating the dynamics of energy input and allocation to organismal processes of importance for characterizing/predicting the condition of the individual (ie. growth and maturation) as well as population-level responses (ie. allocation of energy to reproduction). DEB modeling has great potential for integrating suborganismal processes into individual and population level outcomes (Ananthasubramaniam et al 2015) and could serve to integrate data from dose-responsive relationships among PPARalpha antagonistic nitrotoluenes and fatty acid metabolism, muscle loss and body weight loss (Rawat et al 2010, Deng et al 2011, Wilbanks et al 2014, Quinn et al 2007, Xu and Jin 2012) thus supporting development of a semi-quantitative or quantitative AOP.

Considerations for Potential Applications of the AOP (optional)

The present AOP may require additional conditions to be fully manifested. The risk for this AOP is expected to be exacerbated during fasting, starvation and/or sub-optimal nutrition where interference with PPARα signaling is likely to contribute synergistically toward decreased exercise performance and increased body-weight loss.

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Appendix 1

List of MIEs in this AOP

Event: 998: Binding of antagonist, PPAR alpha

Short Name: Binding of antagonist, PPAR alpha

Key Event Component

Process	Object	Action
receptor antagonist activity	peroxisome proliferator-activated receptor alpha	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:6 - Antagonist binding to PPARa leading to body-weight loss	MolecularInitiatingEvent

Stressors

Name
GW6471

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

Antagonist binding of GW6471 causing increased stabilization of the co-repressors SMRT and N-CoR to the PPARα ligand binding domain has been explicitly demonstrated through x-ray crystallography (Xu et al 2002).

GW6471

Binding to PPAR alpha is implied given observations of inhibited PPAR alpha nuclear signaling inhibition by nitrotoluenes (Wilbanks et al 2014).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
Saccharomyces cerevisiae	Saccharomyces cerevisiae	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	

Sex Applicability

Sex	Evidence
Unspecific	

The fundamental mechanics for nuclear receptor binding as well as demonstration of co-repressor recruitment have been observed to be conserved when comparing humans and yeast (Nagy et al 1999). PPARα has been cloned from frogs, rats, guinea pigs, and humans where the DNA-binding domain has been shown to be identical across species, however the ligand binding domain has exhibited lower homology, likely adapted to differences in dietary ligands among species (Willson et al 2000). Overall, there is evidence for fairly conserved taxonomic applicability across vertebrates, though care should be given when extrapolating across species.

Key Event Description

Binding of molecules to peroxisome proliferator-activated receptor α (PPARα) can cause either agonistic or antagonistic signaling depending on molecular structure (Xu et al 2001, Xu et al 2002). Certain molecules that can bind to the PPARα ligand binding domain have been observed to cause conformational changes that induce increased affinity to co-repressors which decrease PPARα nuclear signaling (Xu et al 2002). Binding of co-repressors such as the silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressor (N-CoR) to PPARα is reinforced by the antagonist, which blocks the AF-2 helix from adopting the active conformation, as demonstrated in x-ray crystallography results presented in Xu et al (2002). Thus, molecules that bind to PPARα that can enhance co-repressor binding act as PPARα antagonists.

How it is Measured or Detected

In Xu et al (2002), X-ray crystallography was used to characterize the suppressed PPARα signaling complex (PPARα / GW6471 / SMRT) and was compared against the activated PPARα complex which included binding of PPARα with the agonist GW409544 and the co-activator, steroid receptor coactivator-1 (SRC-1). For simple PPARα binding assessment, competitive binding assays are availables, however these must be coupled with nuclear signaling activation / inhibition assays to determine if chemicals are agonists or antagonists.

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List of Key Events in the AOP

Event: 1000: stabilization, PPAR alpha co-repressor

Short Name: stabilization, PPAR alpha co-repressor

Key Event Component

Process	Object	Action
transcription corepressor activity	nuclear receptor corepressor 2	increased
transcription corepressor activity	nuclear receptor corepressor 1	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:6 - Antagonist binding to PPARa leading to body-weight loss	KeyEvent

Stressors

Name
GW6471

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
yeast	Saccharomyces cerevisiae	Moderate	NCBI

The fundamental mechanics for co-repressor antagonism of nuclear signaling by SMRT and N-CoR have been observed to be equivalent when comparing humans and yeast (Nagy et al 1999). Therefore, the taxonomic applicability should be broad across eukaryotes. However, the mechanistic perspective for SMRT, N-CoR and PPARα interactions described above was developed exclusively with the human PPARα system. Also, the PPARα nuclear signaling in response to 2,4-DNT and 2A-DNT was established in a human *in vitro* system.

Key Event Description

The transcription co-repressors, silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressor (N-CoR) have been observed to compete with transcriptional co-activators for binding to nuclear receptors (including

PPARα) thus suppressing basal transcriptional activity (Nagy et al 1999, Xu et al 2002). Binding motifs for the co-repressors and co-activators to PPARα have been observed to be conserved, however the co-repressor blocks the AF-2 helix from adopting the active conformation, as demonstrated in x-ray crystallography results presented in Xu et al (2002). PPARα agonists and antagonists have been developed to selectively enhance co-activator or co-repressor binding, respectively (Xu et al 2001, Xu et al 2002). Regarding the present key event, the prior binding of a PPARα antagonists such as GW6471 can stabilize the binding of the co-repressors to the PPARα signaling complex suppressing nuclear signaling and thus downstream transcription of PPARα-regulated genes. Additionally, a natural human variant (V227A) in the hinge region of PPARα has been demonstrated to stabilize PPARα/N-CoR interactions resulting in inhibited transactivation of downstream genes in hepatic cells (Liu et al 2008) which can ultimately lead to measurable phenotypic effects of decreased blood triglycerides (Chan et al 2016). The Liu et al (2008) study also demonstrated that the transactivation activity in the V227A variant could be restored when N-CoR was silenced.

Wilbanks et al. (2014) and Gust et al (2015) demonstrated inhibition of human PPARα nuclear signaling in *in vitro* nuclear signaling bioassays in response to 2,4-dinitrotoluene(2,4-DNT) and 2-amino-4,6-dinitrotoluene (2A-DNT), respectively. However, it is unknown if this response was manifested through the co-repressor binding stabilization that was identified in Xu et al (2002).

How it is Measured or Detected

In Xu et al (2002), X-ray crystallography was used to characterize the suppressed PPARα signaling complex (PPARα / GW6471 / SMRT) and was compared against the activated PPARα complex which included binding of PPARα with the agonist GW409544 and the co-activator, steroid receptor coactivator-1 (SRC-1). Liu et al (2008) conducted in vitro investigations comparing human PPARα WT versus the V227A mutant in cell-based assays evaluated using quantitative real-time RT-PCR, competitive binding assays, immunofluorescence, GST pulldown, ChIP, immunoprecipitation. In Chan et al (2006) triglycerides were measured directly from human blood serum samples from populations having the wild type PPARα gene compared to those having the V227A variant genotype.

The effects of 2,4-DNT and 2A-DNT on PPAR signaling was investigated using nuclear receptor reporter assays were conducted for (PPARalpha, PPARgamma, PPARsigma, and RXRalpha human cell-based assays, (Indigo Biosciences).

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Event: 858: Decreased, PPARalpha transactivation of gene expression

Short Name: Decreased, PPARalpha transactivation of gene expression

Key Event Component

Process	Object	Action
receptor transactivation	peroxisome proliferator-activated receptor alpha	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:6 - Antagonist binding to PPARa leading to body-weight loss	KeyEvent

Stressors

Name
PPARalpha antagonists

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Not Specified

Sex Applicability

Sex	Evidence
Male	High
Female	High

Mus musculus (Kersten 2014), *Homo sapiens* in clinical observations (Kersten 2014) and in in vitro assays (reviewed in Kersten 2014). Aspects of PPARα signaling have been observed to differ comparing human and rodent responses. Microarray-based comparative transcriptomic expression among mouse and human primary hepatocyte samples exposed to the PPARα agonist Wy14643 showed minor overlap in individual gene-level expression, however substantial overlap was observed at the pathway level (Rakshanderhroo et al 2009). In that study, most of the genes found to have differential expression in common among human and mouse were involved in lipid metabolism, including CPT1A, HMGCS2, FABP1, ACSL1 and ADFP. Thus, in Rakshanderhroo et al (2009), evaluation of PPARα transactivation differences among human and mouse suggest expression for gene-transcripts involved in lipid metabolism tended to be the most conserved among species. (IMPORTANT NOTE: The results from Rakshanderhroo et al (2009) should be viewed with caution given a potential an N=1 for all of the mouse strains where primary hepatocytes were obtained from 1 mouse per strain.) Feige et al (2010) found that mice with humanized PPARα were insensitive to the PPARα agonist pollutant, diethylhexyl phthalate (DEHP), where mRNA expression (measured by reverse transcriptase-qPCR) for genes involved in fatty-acid metabolism were not induced relative to wild type mice. (IMPORTANT NOTE: the results from Fiege et al (2010) show that DEHP is a weak partial agonist of PPARα. Additionally, the use of wild type versus mice with humanized PPARα for extrapolating species-to-species differences should be viewed with caution. Humanized receptor is not likely to interact with the same cofactors in mice

relative to humans and the regulatory grammars may differ between among species that may further complicate the biochemistry.)

Key Event Description

PPARα acts as a nuclear signaling element that controls the transcription of a variety of genes involved in lipid catabolism and energy production pathways (Desvergne and Wahli 1999, Kersten 2014). Fatty acids serve as the ligands that stimulate PPARα nuclear signaling where the fatty acids (likely in association with fatty acid binding proteins) bind to the ligand binding domain of PPARα along with co-activators to the PPARα regulatory complex at promoter regions of PPARα-regulated genes (termed PPAR response elements, PPREs) initiating the transcription of genes that metabolize the fatty acids (Ahmed et al 2007, Wolfrum et al. 2001, Desvergne and Wahli 1999, Kersten 2014, Xu et al 2001, Janssen et al 2015).

Specifically, PPARα contains both a ligand-binding domain that binds fatty acids and a DNA-binding domain that initiates binding to PPREs in the promoter regions of PPARα-regulated genes (Ahmed et al 2007, Hihi et al 2002). Binding of the fatty acid ligands to PPARα facilitates heterodimeric binding with another ligand-activated nuclear receptor, the retinoid X receptor (RXR), forming an activated PPAR-RXR transcriptional regulator complex (DiRenzo et al 1997, Ahmed et al 2007). PPAR competes for binding to RXR with retinoic acid receptors (RARs) where the RAR/RXR heterodimer inhibits transcription of genes downstream of PPREs (DiRenzo et al 1997). Transcriptional regulation activity of the PPAR/RXR complex is also influenced by the binding and release of accessory molecules that act as coactivators such as steroid receptor co-activator 1 (SRC-1) or as corepressors such as nuclear receptor corepressor (N-CoR, DiRenzo et al 1997, Ahmed et al 2007, Xu et al 2002, Liu et al 2008). Such binding of the co-repressor N-CoR to the PPARα/RXRα complex has been demonstrated to inhibit the potential for transcriptional transactivation (Xu et al 2002, Liu et al 2008). The exact mechanisms by which the PPAR/RXR complex facilitate transcription are still not well understood. It has been observed that RXR contains a highly conserved motif at the C-terminal end of the ligand-binding domain known as activating function 2 (AF2) which undergoes conformational changes allowing interaction with coactivators / corepressors, the former of which is hypothesized to recruit the components of the transcriptional machinery necessary to transcribe the downstream gene (DiRenzo et al 1997). Even the basal transcriptional machinery itself is recognized to vary across cell types and the prototypical preinitiation complex (PIC) is inherently highly flexible, confirmationally diverse including multi-faceted interactions of activators, core promotion factors, the RNA polymerase II enzyme, elongation factors, and chromatin remodeling complexes all combined at the promoter to facilitate gene transcription (Levine et al 2014). Additionally, recent transcriptomic research coupling transcriptomic expression and chromatin immunoprecipitation (ChIP) sequencing to identify PPARα binding to PPREs suggests PPARα may exert transcriptional regulation beyond its direct genomic targets via secondary signaling networks including various kinases (McMullen et al 2014). Given the current KE (KE2, PPARalpha transactivation of gene expression, Decreased), a variety of upstream influences may impair the function of the PPARα-RXRα heterodimer and/or affect coactivator / corepressor binding leading to decreased PIC competence resulting in impaired transcription of downstream genes as well as secondary signaling networks.

PPARα regulates expression of genes encoding nearly every enzymatic step of fatty acid catabolism including fatty acid uptake into cells, fatty acid activation to acyl-CoAs, and the release of cellular energy from fatty acids through the oxidative breakdown of acyl-CoAs to acetyl-CoA, and in starvation conditions, the repackaging of Acetyl-CoA substrates into ketone bodies via ketogenesis pathways (Kersten 2014, Desvergne and Wahli 1999, Evans et al 2004). A pathway-level schematic for PPARα transactivation is illustrated in KEGG Pathway map03320 providing the specific gene targets and associated functional responses that are transcriptionally regulated by PPARα. It should be noted that there are species-specific differences in PPARα transactivation of gene expression among mice and humans which are explained in the "Evidence Supporting Taxonomic Applicability" section, below.

How it is Measured or Detected

X-ray crystallography was used to describe the ligand binding domain (fatty acid binding domain) of PPARα and demonstrate the binding complex of PPARα, the artificial ligand (GW6471), and the co-repressor silencing mediator for retinoid and thyroid hormone receptors (SMRT, Xu et al 2001, 2002). Fold activation of the PPARα-GW6471-SMRT transcriptional regulatory complex was measured in mammalian two-hybrid assays (Xu et al 2001). PPAR-RXR and RAR-RXR heterodimerization and activity were quantified using expression vectors for murine PPARα, RAR and RXR in CV-1 cells transfected with 1ug of reporter plasmid and 50-200ng of expression plasmid (DiRenzo et al 1997). DNA-dependent radioligand binding assays were conducted to quantify ligand binding in the assays described in DiRenzo et al (1997).versions of each individual. X-ray crystallography has been used with limited success to describe the PIC while high-resolution electron microscopy is providing additional insights into the fully functionalized PIC when bound to the promotor and including all accessory molecules (Levine et al 2014). Effects of PPARα transactivation on expression of downstream genes been examined by a variety of methods, especially RT-qPCR (Kersten et al 2014). As a recommendation for investigating specific genes regulated by PPARα, as part of this KE, see the KEGG pathway for PPAR Signaling (map03320). In McMullen et al (2014), transcriptomic expression was investigated in human primary hepatocytes in time and dose series exposures to the PPARα agonist GW7647 where transcriptomic expression was measured using Affimetrix microarrays and ChIPseq was conducted after completing immunoprecipitation and quantified using Illumina HiSeq 2000 sequencing to find binding to PPRE within 50K bp of differentially expressed genes.

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Event: 1528: Fatty Acid Beta Oxidation, Decreased

Short Name: Fatty Acid Beta Oxidation, Decreased

Key Event Component

Process	Object	Action
fatty acid beta-oxidation		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:6 - Antagonist binding to PPARa leading to body-weight loss	KeyEvent

Stressors

Name
PPARalpha antagonists

Biological Context

Level of Biological Organization
Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Human (as reviewed in Brandt et al 1998, Evans et al 2004, Gulick et al 1994, Kersten 2014 and Desvergne and Wahli 1999; various product labels for fibrate drugs available at the US FDA). Rat (as measured by Lazarow 1978). Mouse (as reviewed in Kersten 2014 and Desvergne and Wahli 1999). Fatty acid oxidation in response to various PPAR agonists administered in mouse and human skeletal muscle tissues using *in vitro* assays showed the same response profiles among species (Muoio et al 2002). Results from Rakshanderhroo et al (2009) showed that PPARα transactivation caused unique transcriptional expression profiles between human and mouse, however expression for genes involved in lipid metabolism tended to be the most conserved among species. (IMPORTANT NOTE: The results from Rakshanderhroo et al (2009) should be viewed with caution given a potential an N=1 for all of the mouse strains where primary hepatocytes were obtained from 1 mouse per strain.) Interestingly, PPARα-humanized mice exposed to the PPARα agonist Wy-14643 showed no hepatocellular proliferation compared to wild type mice, but both humanized and wild types showed the same capacity for induction of peroxisomal and mitochondrial fatty acid metabolizing enzymes and resultant decreases of blood-serum trigycerides (Cheung et al 2004). (IMPORTANT NOTE: The use of wild type versus mice with humanized PPARα for extrapolating species-to-species differences should be viewed with caution. Humanized receptor is not likely to interact with the same cofactors in mice relative to humans and the regulatory grammars may differ between among species that may further complicate the biochemistry.)

Key Event Description

PPARα acts as a positive transcriptional regulator for many of the genes involved in peroxisomal and mitochondrial fatty acid beta oxidation as well as genes involved in the pre- and post-processing of fatty acids in peroxisomal pathways (Desvergne and Wahili 1999, Kersten 2014). Thus, decreased PPARα nuclear signaling results in decreased transcriptional expression of genes that are regulated by PPARα, and subsequently, decreased expression of the coded proteins and enzymes that ultimately decreased fatty acid metabolism within peroxisomes and mitochondria. In PPARα knock-out mice, fatty acid oxidation capacity was decreased in well fed, rested mice and the reduced fatty acid oxidation capacity was greatly exacerbated during exercise challenge or 24h starvation (Muoio et al 2002).

Genes Involved: The first gene target identified for PPARα was Acyl-CoA oxidase (Acox1, Dreyer et al 1992) which represents the first enzyme in peroxisomal long-chain fatty acid oxidation (Kersten 2014) and is also the rate-limiting enzyme in this pathway (Desvergne and Wahili 1999). In addition to Acox1, a variety of additional enzymes involved in peroxisomal fatty acid metabolism are under transcriptional control of PPARα transactivation including enzymes that facilitate fatty acid uptake into the peroxisome (Abcd1, Abcd2 and Abcd 3), conversion of acyl-CoA/acetyl-CoA to acyl-carnitine/acetyl-carnitine (Crot/Crat), and conversion of acyl-CoAs back to fatty acids via thioesterases (Acots, as reviewed in Kersten 2014). PPARalpha also has transcriptional control over enzymes downstream of Acox1 in the peroxisomal beta-oxidation of acyl-CoA pathway including L-bifunctional enzyme (Ehhadh), D-bifunctional enzyme (Hsd17b4), and peroxisomal 3-ketoacyl-CoA thiolase activity (Acaa1a, Acaa1b, as reviewed in Kersten 2014). All of these genes are potential targets for screening affects within this KE.

As reviewed in Kersten (2014), the genes (and associated functions) regulated by PPARα in the mitochondrial processing of fatty acids include the following: (1) Import of acyl-CoAs into the mitochondria is facilitated by PPARα-induced increases in expression of carnitine palmitoyl-transferases 1a, 1b, and 1 (Cpt1a, Cpt1b, Cpt2) and acyl-carnitine translocase (Slc25a20; Brandt et al 1998, Mascaro et al 1998, Feige et al 2010). (2) The first step of mitochondrial beta-oxidation is catalyzed by length-specific acyl-CoA hydrogenases (Acadvl, Acadl, Acadm, Acads; Aoyama et al 1998, Gulick et al 1994). (3) The three subsequent steps in mitochondrial beta-oxidation that successively release acetyl-CoAs from the hydrocarbon chain are catalyzed by the mitochondrial trifunctional enzyme (Hadha and Hadhb). These enzymes are replaced upon progressive chain shortening by Hadh and Acaa2. (4) the final PPARalpha targets include Eci1, Eci2, Decr1, and Hsd17b10 which convert unsaturated and 2-methlylated aclyl-CoAs into intermediates of beta-oxidation (Sanderson et al 2008, Aoyama et al 1998).

Metabolism Affected: Peroxisomes participate in a variety of lipid metabolic pathways including the beta-oxidation of very long-straight chain (<20 C in length) or branched – chain acyl-CoAs (Lazarow 1978, Kersten 2014). The peroxisomal beta-oxidation pathway is not directly coupled to the electron transport chain and oxidative phosporylation, therefore the first oxidation reaction loses energy to heat (H2O2 production) while in the second step, energy is captured in the metabolically accessible form of high-energy electrons in NADH (Mannaerts and Van Veldhoven 1993, Desvergne and Wahli 1999). The peroxisomal beta-oxidation pathway provides fatty acid chain shortening where two carbons are removed in each round of oxidation in the form of acetyl-CoA (Desvergne and Wahli 1999). The acetyl-CoA monomers serve as fundamental units for metabolic energy production (ATP) via the citric acid cycle followed by electron-transport chain mediated oxidative phosphorylation (Nelson and Cox, 2000A) as well as serve as the fundamental units for energy storage via gluconeogenesis (Nelson and Cox, 2000B) and lipogenesis (Nelson and Cox, 2000C). The shortened chain fatty acids (<20C) can then be transported to the mitochondria to undergo mitochondrial beta-oxidation for complete metabolism of the carbon substrate for cellular energy production (Desvergne and Wahli 1999).

Mitochondrial processing of fatty acids involves: (1) Import of short, medium and long chain fatty acids (<C20) acyl-CoAs into the mitochondria by carnitine palmitoyl-transferases 1a, 1b, and 1 (Cpt1a, Cpt1b, Cpt2) and acyl-carnitine translocase (Slc25a20, Brandt et al 1998; Mascaro et al 1998, Kersten et al 2014). (2) The first step of beta-oxidation catalyzed by the length-specific acyl-CoA hydrogenases (Acadvl, Acadl, Acadm, Acads; Aoyama et al 1998, Gulick et al 1994, Kersten et al 2014). (3) The three subsequent steps in mitochondrial beta-oxidation that successively release acetyl-CoAs from the hydrocarbon chain are catalyzed by the mitochondrial trifunctional enzyme (Hadha and Hadhb, Kersten et al 2014). These enzymes are replaced upon progressive chain shortening by Hadh and Acaa2 (Kersten et al 2014). (4) The conversion of unsaturated and 2-methylated acetyl-CoAs into intermediates of beta-oxidation are catalyzed by Eci1, Eci2, Decr1, and Hsd17b10 (Sanderson et al 2008, Aoyama et al 1998, Kersten et al 2014). Interestingly, tissue-specific differences in sensitivity to PPARα knock out have been observed in mice where fatty acid oxidation was markedly impaired in liver and heart tissues whereas skeletal muscle was largely unaffected due to seven-fold increased

abundance of PPARδ in skeletal muscle tissue (Muoio et al 2007). Redundancy in regulation of genes involved in fatty acid metabolism across PPARα, PPARβ, PPARδ, and PPARγ are known in canonical signaling pathways (KEGG Pathway map03320) where metabolic responses are coordinated across the iso-types with whole-organism-level effects on insulin sensitivity, body fat, and body weight (Harrington et al 2007).

How it is Measured or Detected

Beta oxidation of fatty acids in mitochondria was measured using mouse liver homogenates where a radio-labeled fatty acid substrate was reacted for 30 minutes and then centrifuged to separate reaction products for fractional radioactivity measurements in Aoyama et al (1998). Comparative measures of reaction products were also measured where potassium cyanide was added to the reaction mixture to inhibit mitochondrial beta oxidation activity to normalize the contribution of mitochondrial enzymatic reactions to the overall reaction product (Aoyama et al 1998). In Muoio et al (2002), capacity for beta oxidation of fatty acids in serum was determined by measuring the nonesterified fatty acids and transcript expression was quantified using RT-qPCR. Harrington et al (2007) administered agonists for the various PPARs and PPARpan (affecting all PPARs simultaneously) used *ex vivo* quantification of PPAR-induced fatty acid oxidation of ^{14}C-labeled fatty acids to CO_2.

A variety of transcript expression assays have been used to demonstrate the effect of PPARα signaling inhibition on downstream transcript expression (see literature cited above for specific methods within each investigation). Investigation of PPARα transcriptional targets (especially those involved in fatty acid metabolism) have been conducted via variety of methods, with RT-qPCR being the benchmark standard (Kersten 2014 and Feige et al 2010). Spectroscopic analysis of the characteristic absorption bands for fatty acid substrates and fatty acid beta oxidation products were examined for peroxisomal fractions purified from rat livers by differential and of equilibrium density centrifugation (Lazarow 1978). Additionally, NAD reduction assays were conducted for acyl-CoA substrates with varying chain lengths where increased oxidation was observed for substrates with long chain length relative to short chain acyl-CoAs (Lazarow 1978).

Brandt et al (1998) investigated concentration response effects of Oleate, Decanoate and Hexanoate fatty acid chains on mitochondrial carnitine palmitoyl-transferases I (M-CPT I) expression using promoter-reporter plasmid MCPT.Luc.1025 reporter transfected into rat neonate cardiac myocytes. Human M-CPT I was investigate using an analogous method (Brandt et al 1998). Expression of human medium chain acyl-CoA dehydrogenase (MCAD) was investigated using a MCAD.luc.1054 reporter transfected into HepG2 cells in response to fatty acids with various chain lengths (Gulick et al 1994). Investigation of various enzymes involved in hepatic fatty acid metabolism described in Aoyama et al (1998) were investigated using Western immunoblot quantitiation.

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Event: 861: Decreased, Ketogenesis (production of ketone bodies)

Short Name: Decreased, Ketogenesis (production of ketone bodies)

Key Event Component

Process	Object	Action
ketosis		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:6 - Antagonist binding to PPARa leading to body-weight loss	KeyEvent

Stressors

Name
PPARalpha antagonists

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Not Specified
Adults	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Evidence provided for human in Cahill (2006), Owen et al (2005) and Williamson et al (1962). Evidence for mouse provided in Kersten et al (1999). Comparative investigations of ketone body formation comparing human and mouse is not well established relative to fatty-acid oxidation comparisons.

Key Event Description

PPARα acts as a positive transcriptional regulator for many of the genes involved in ketogenesis (Desvergne and Wahili 1999, Kersten 2014). Thus, decreased PPARα nuclear signaling results in decreased transcriptional expression of genes that are regulated by PPARα, and subsequently, decreased expression of the coded proteins and enzymes that ultimately impair ketogenesis. Ketogenesis is critical to supporting general systemic energy homeostasis in fasting events (Cahill 2006, Evans et al 2004, Sengupta et al 2010), thus KE4 becomes important after short term energy stores (glycogen) become limited (Muoio et al 2002). Le May et al (2000) have shown decreased ketogenesis in livers of PPAR null mice linked to impaired mitochondrial hydroxymethylglutaryl-CoA synthase (Hmgcs) gene expression.

Genes Involved: Not only does PPARα regulate the genes that catalyze the upstream production of the raw materials utilized in ketogenesis through fatty acid beta-oxidation (see decreased peroxisomal (KE3) and mitochondrial (KE4) fatty acid beta oxidation, upstream), but also directly induces key enzymes in the ketogenesis pathway including Hmgcs2, Hmgcl, and Acat1 (Kersten et al 2014, Le May et al 2000). PPARα is recognized as the master transcriptional activator of ketogenic genes (Sengupta et al 2010, Desvergne and Wahli 1999).

Metabolism Affected: The liver plays a key role in processing the fundamental energy substrate, acetyl-CoA, into metabolic currencies that contribute to the systemic cellular energy needs of the whole organism. The liver represents a key organ involved in systemic energy distribution given its ability to synthesize glucose (an ability shared only with the kidney, Gerich et al 2001) as well as its exclusive role in the generation of ketone bodies (Cahill 2006, Sengupta et al 2010, Kersten 2014). This is especially important for the metabolic energy needs of the brain which can only use glucose and the ketone body, β-hydroxybutyrate for cellular energy production (Cahill 2006, Owen 2005, Kersten 2014). Therefore, ketogenesis is critical to supporting general systemic energy

homeostasis in fasting events (Cahill 2006, Evans et al 2004, Sengupta et al 2010). Interference with ketogenesis, for example by PPARα inhibition, has been demonstrated to inhibit β-hydroxybutyrate production (measured in serum) during fasting events in mice (Le May et al 2000, Badman et al 2007, Potthoff 2009, Sengupta et al 2010) and cause hypoketonemia (Muoio et al 2002). The Badman et al (2007) study indicated that metabolism of fatty acid substrates (measured as liver triglycerides) that would otherwise contribute to β-hydroxybutyrate production was additionally inhibited under PPARα knockout.

In a fasting state, humans transition from the use of exogenous glucose to glucose derived from glycogen within 4 hours with a steadily increasing proportion of glucose usage that is derived from gluconeogenesis up to 2 days (Cahill 2006). Beyond 2 days of fasting, ketone body production (β-hydroxybutyrate) increasingly supports the energy demands of the brain (Cahill 2006).

How it is Measured or Detected

The quantification of β-hydroxybutyrate described in Cahill 2006 was measured in a cell-free system catalyzed by D(-)-p-hydroxybutyric dehydrogenase where all components of the reaction [D(-)-fl-hydroxybutyrate + DPN+ = acetoacetate + DPNH + H+] were able to be quantitatively determined (Williamson et al 1962). Serum β-hydroxybutyrate was measured using Stanbio Laboratory small-scale enzymatic assays in Badman et al (2007) and by Wako Chemicals D-3-hydroxybutyric acid kit in Potthoff et al (2009). SMART micro-FPLC (Amersham Biosciences) consisting of a Superose 6 PC 3.2/30 column (Amersham Biosciences) equilibrated in 13 PBS buffer was conducted where triglyceride and cholesterol fractions were investigated by enzymatic assay (Wako Diagnostics) as described in Badman et al (2007). Clinical observations of ketone bodies have been simplified by the development of urine test strips that can provide quantitative values for the ketone bodies aceto-acetate, acetone and 3-hydroxybutyrate using reflectometry (Penders et al 2005). In Le May et al (2000), glucose, L-hydroxybutyrate and acetoacetate concentrations were measured in the neutralized perchloric filtrates by enzymatic methods. In Muoio et al (2002), β-hydroxybutyrate was measured in blood serum comparing wild type and PPARα knockout mice.

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Event: 862: Not Increased, Circulating Ketone Bodies

Short Name: Not Increased, Circulating Ketone Bodies

Key Event Component

Process	Object	Action
abnormal lipid homeostasis		abnormal
	ketone body	abnormal

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:6 - Antagonist binding to PPARa leading to body-weight loss	KeyEvent

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
blood

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Not Specified
Adults	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Evidence for mouse provided in (Badman et al 2007, Potthoff 2009). Evidence for human provided in (Cahill 2006, Owen et al 2005, Gerich et al 2001). Comparative investigations of ketone body formation comparing human and mouse is not well established relative to fatty-acid oxidation comparisons.

Key Event Description

A fundamental process in biological systems is the production of metabolic fuel for use in meeting the energy demands of cells and, in multi-cellular organisms, supporting overall systemic energy needs. Physiological studies of the progression of human starvation have identified that the preferred metabolic fuel is glucose in the fed state and progressing through two days of fasting, afterward ketone bodies become increasingly important for meeting energy demands (Cahill 2006, Owen et al 2005). Substrates derived from

carbohydrates, fats and protein can contribute to gluconeogenesis (Cahill 2006, Gerich et al 2001) whereas substrates derived from fatty acids are the primary contributors to ketogenesis (Desvergne and Wahli 1999). Mobilization of fatty acids as a metabolic fuel source increase dramatically during fasting to support both gluconeogenesis and ketogenesis (Evans et al 2004). Cahill (2006) and colleagues have demonstrated the importance of ketone body production, especially β-hydroxybutyrate, for maintaining energy homeostasis during starvation. β-hydroxybutyrate serves as an alternative substrate to glucose for providing energy to the brain in the starvation state, providing ATP at higher efficiency relative to the glucose substrate (Cahill 2006). Interference with ketogenesis, for example by PPARα inhibition, has been demonstrated to inhibit β-hydroxybutyrate production (measured in serum) during fasting events in mice (Badman et al 2007, Potthoff 2009, Sengupta et al 2010) and cause hypoketonemia (Muoio et al 2002). The Badman et al (2007) study indicated that metabolism of fatty acid substrates (measured as liver triglycerides) that would otherwise contribute to β-hydroxybutyrate production was inhibited under PPARα knockout. Increased concentrations of circulating ketone bodies is indicative of potential metabolic fuel deficits in fasting animals (Cahill 2006), and a lack of increase in circulating ketone bodies during fasting, especially in conjunction with elevated blood triglycerides, indicates impaired ketogenesis and potentially impaired bioenergetic potential. Although the potential therapeutic implications of increased ketone body metabolism via ketogenic diets for various disease states has been discussed (Veech 2004), no studies were found demonstrating effects on whole organism responses to impaired ketogenesis over long-term starvation events. A potential implication of decreased ketone body production is stress on cardiac function given that energy-stressed heart tissue shifts reliance away from fatty acids toward ketone bodies (β-hydroxybutyrate) to fuel production of the ATP needed to maintain the heart's mechanical function (Aubert et al 2016). Related to this observation, PPARα-knockout mice reached exhaustion sooner than wild types in an exercise challenge which corresponded with significantly decreased β-hydroxybutyrate in serum indicating hypoketonemia in PPARα-knockout mice versus wild types (Muoio et al 2002). Overall, diminished PPARα function, especially in combination with fasting /diminished nutrition and/or excessive exercise may contribute to impaired maintenance on systemic energy budget.

How it is Measured or Detected

The quantification of β-hydroxybutyrate described in Cahill 2006 was measured in a cell-free system catalyzed by D(-)-p-hydroxybutyric dehydrogenase where all components of the reaction [D(-)-fl-hydroxybutyrate + DPN+ = acetoacetate + DPNH + H+] were able to be quantitatively determined (Williamson et al 1962). Serum β-hydroxybutyrate was measured using Stanbio Laboratory small-scale enzymatic assays in Badman et al (2007) and by Wako Chemicals D-3-hydroxybutyric acid kit in Potthoff et al (2009). SMART micro-FPLC (Amersham Biosciences) consisting of a Superose 6 PC 3.2/30 column (Amersham Biosciences) equilibrated in 13 PBS buffer was conducted where triglyceride and cholesterol fractions were investigated by enzymatic assay (Wako Diagnostics) as described in Badman et al (2007). Clinical observations of ketone bodies have been simplified by the development of urine test strips that can provide quantitative values for the ketone bodies aceto-acetate, acetone and 3-hydroxybutyrate using reflectometry (Penders et al 2005). The transition from using fatty acids to ketone bodies to fuel ATP production in cardiac muscle was measured in isolated heart preparation using *ex vivo* NMR combined with targeted quantitative myocardial metabolomic profiling using mass

spectrometry (Aubert et al 2016). In Muoio et al (2002), β-hydroxybutyrate was measured in blood serum comparing

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Event: 863: Increased, Catabolism of Muscle Protein

Short Name: Increased, Catabolism of Muscle Protein

Key Event Component

Process	Object	Action
protein catabolic process	muscle protein	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:6 - Antagonist binding to PPARa leading to body-weight loss	KeyEvent

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
musculoskeletal system

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus rattus	Rattus rattus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

Evidence for mouse provided in (Cahill 2006, Marliss et al 1971, Gelig et al 1970A, 1970B). Evidence for rat provided in Kashiwaya et al 1994, Goodman et al 1966). Evidence for human provided in (Cahill 2006).

Key Event Description

After two to three days of fasting in humans, dietary glucose has been long-since expended and contribution to blood glucose from glycogen metabolism is reduced to zero (Cahill 2006). At this point, about two fifths of fatty acid metabolism in the whole body is dedicated to hepatic ketogenesis, largely in support of the energy demands of the brain, however the brain is still significantly supported by glucose derived from gluconeogenesis (Cahill 2006). PPARα knockout mice that were either fasted or exercised to exhaustion had diminished capacity for maintaining energetic substrates in serum (glucose and lactate) while showing diminished capacity for fatty acid oxidation (serum nonesterified fatty acids) and decreased ketogenesis resulting in hypoketonemia (decreased serum β-hydroxybutyrate) relative to wild types (Muoio et al 2002). As fatty acid stores are depleted or become unusable (as in the PPARα knockout condition described above), gluconeogenesis from other substrates becomes increasingly important including muscle protein catabolism *in situ* for supporting muscle function as well as releasing glutamine (Marliss et al 1971) and alanine (Felig et al 1970A) which can be recycled to glucose by gluconeogenesis in the kidney (Goodman et al 1966, Kashiwaya et al 1994, Cahill 2006). Renal gluconeogenesis from glutamine and alanine supports two fifths of new glucose production while the remaining three fifths is produced in liver from, (a) alanine derived from muscle and nonhepatic splanchnic bed, (b) recycled lactate and pyruvate from red blood cells and renal medulla, (c) glycerol from adipose lipolysis and (d) small amounts of β-hydroxybutyrate are recycled to glucose (Cahill 2006). Blood concentrations of alanine exert control over hepatic glucose production and thus also represent a diagnostic of alanine contribution from muscle to support gluconeogenesis (Cahill 2006, Felig et al 1970B). In prolonged starvation events, the catabolism of muscle protein for gluconeogenesis in order to support systemic energy needs results in loss of muscle mass which contributes to loss of overall body weight. Although it has not yet been investigated experimentally, it is plausible based on the results described above for Muoio et al (2002) that diminished PPARα signaling capacity could exacerbate muscle wasting in long-term fasting and/or malnutrition events.

How it is Measured or Detected

Glutamate and glutamine were measured in fresh plasma taken from human subjects that were fasted and those in a postabsorptive state using enzymatic assays (Mariliss et al 1971). In Kashiwaya et al (1994), perfused rat hearts were prepared for metabolic flux experiments. Measurement of enzyme kinetics involved in glycoloysis and gluconeogenesis were measured using fluorometric procedures measuring the oxidation or reduction of pyridine nucleotides. Radio-labeled substrates were used to track metabolite flux during glucolysis / gluconeogenesis. Goodman et al provided in vitro assessment of gluconeogenic capacity of renal cortex in rats. Glutamatic acid and other ketogenic substrates were added and measure in the system and measured as net glucose content. All amino acids were measured in Felig et al (1970A), however the analytical methods that were references were not found using Google Scholar search. In Muoio et al (2002), blood

glucose, lactate and β-hydroxybutyrate were measured in blood serum while capacity for beta oxidation of fatty acids was determined by measuring the nonesterified fatty acids in blood serum.

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List of Adverse Outcomes in this AOP

Event: 864: Decreased, Body Weight

Short Name: Decreased, Body Weight

Key Event Component

Process	Object	Action
weight loss		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:6 - Antagonist binding to PPARa leading to body-weight loss	AdverseOutcome

Biological Context

Level of Biological Organization
Individual

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI
Mus musculus	Mus musculus	High	NCBI
Colinus virginianus	Colinus virginianus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	Moderate

Sex Applicability

Sex	Evidence
Male	High
Female	High

Evidence for human (Kersten 2014, Evans et al 2004, Desvergne and Wahli 1999). Evidence for mice (Badman et al 2007, Sanderson et al 2010, Wilbanks et al 2014, Xu et al 2012, Kersten et al 1999). Evidence for birds (Martin et al 1987).

Key Event Description

If caloric intake is less than caloric use over time, an individual will lose body weight. This is a basic principle in human dieting as well as an important principle related to individual health and ecological fitness of animal populations. Dynamic energy budget theory has provided useful insights on how organisms take up, assimilate and then allocate energy to various fundamental biological processes including maintenance, growth, development and reproduction (Nisbet et al 2000). Regarding energy allocation, somatic maintenance must first be met before then growth may occur, followed by maturation and then finally, surplus energy is dedicated to reproduction (Nisbet et al 2000). As an example of the importance of energy allocation to ecological fitness, a review by Martin et al (1987) demonstrated that energy availability (availability of food) was the predominant limiting factor in reproductive success and survival for both young and parents in a broad life history review for bird species. This is a likely scenario for many organisms.

Various physiological processes act to maintain and prioritize energy allocations in individuals. The influence of PPARalpha on systemic energy metabolism and energy homeostasis has been broadly established (see reviews by Kersten 2014, Evans et al 2004, Desvergne and Wahli 1999). Inhibition of PPARalpha predominantly impairs lipid metabolism with respect to overall energy metabolism whereby energy release from fatty acid substrates is decreased. PPARalpha has been demonstrated to play a critical role in stimulating fatty acid oxidation and ketogenesis during fasting resulting in increased ketone body levels in plasma (Badman et al 2007, Kersten 2014) a response that is eliminated in PPARalpha knockout mice (Badman et al 2007, Sanderson et al 2010). A reviews by Cahill (2006) and Wang et al (2010) summarize the critical adaptive response of ketogenesis during fasting for maintaining systemic energy homeostasis by providing ketone bodies to energetically fuel a diverse range of tissues, especially the brain. Not only does PPARalpha induce the upstream production of the raw materials for use in ketogenesis through fatty acid beta-oxidation, but also directly induces key enzymes in the ketogenesis pathway including Hmgcs2, Hmgcl and Acat1 (Kersten 2014).

Kersten et al (1999) and Badman et al (2007) demonstrated that PPARalpha-null mice were unable to actively mobilize fatty acid oxidation, and further, Kersten et al (1999) demonstrated that these mice were unable to meet energy demands during fasting and leading to hypoglycemia, hyperlipidemia, hypoketonemia and fatty liver. Observations from toxicological and toxicogenomic research have implicated nitrotoluenes as potential PPAR antagonists in birds (Rawat et al 2010), rats (Deng et al 2011) and mice (Wilbanks et al 2014), an effect that additionally corresponded with weight loss in rats (Wilbanks et al 2014) and weight loss, loss of muscle mass and emaciation in birds (Quinn et al 2007). These combined results indicate that inhibition of PPARalpha signaling and the resultant decrease in fatty acid oxidation and ketogenesis can detrimentally impair systemic energy budgets leading to starvation-like effects and resultant weight loss. As reviewed in the introductory paragraph of this adverse outcome description, impaired energy availability leading to inability to meet somatic maintenance needs and causing negative growth are likely to have detrimental effects on survivorship, reproduction and ecological fitness. Such affects may adversely affect responses of regulatory concern including: individual health, survival, and population sustainability.

How it is Measured or Detected

Dynamic energy budget model development and validation demonstrated against various parameter values and population studies (Nisbet et al 2000). Food availability, animal weights, brood sizes, adult survival, and juvenile survival measured in Martin et al (1987). Whole body animal weights were measured for mice in Wilbanks et al (2014). Whole body weights, organ weights and breast muscle weights measured in Quinn et al (2007). In vitro human PPARalpha nuclear-receptor activation/inhibition assays have been used to determine if chemicals interfere with PPARalpha nuclear signaling (Wilbanks et al 2014, Gust et al 2015). Transcript Expression of PPARalpha as well as transcript expression for genes in which PPARalpha acts as a transcriptional regulator (Wilbanks et al 2014, Deng et al 2011, Rawat et al 2010, and studies reviewed in Kersten 2014).

Regulatory Significance of the AO

WWeight loss in wild populations has direct implications on fitness as demonstrated dynamic energy budget modeling (Nisbet et al 2000). Thus weight loss can be used as a metric for populations sustainability. For individuals, rapid weight loss of greater than 20% total body weight is considered indicative of a moribund condition in laboratory animals for many Institutional Animal Care and Use Committees as established by American Association for Laboratory Animal Science (https://www.aalas.org/iacuc#.ViZzMSvaFs).

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Appendix 2

List of Adjacent Key Event Relationships in this AOP

Relationship: 1024: stabilization, PPAR alpha co-repressor leads to Decreased, PPARalpha transactivation of gene expression

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonist binding to PPARα leading to body-weight loss	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
yeast	Saccharomyces cerevisiae	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Not Specified

Sex Applicability

Sex	Evidence
Male	High
Female	High

The majority of the studies cited herein provide evidence for human and rat, however much of the signaling architecture is also present in yeast (Krogsdam et al 2002). However, the mechanistic perspective for SMRT, N-CoR and PPARα interactions described above was developed exclusively with the human PPARα system.

Key Event Relationship Description

The transcription co-repressors, silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressor (N-CoR) have been observed to compete with transcriptional co-activators for binding to nuclear receptors (including PPARα) thus suppressing basal transcriptional activity (Nagy et al 1999, Xu et al 2002). Natural human variant (V227A) in the hinge region of PPARα has also been demonstrated to stabilize PPARα/N-CoR interactions resulting in inhibited transactivation of downstream genes in hepatic cells, a response that was reversed when N-CoR was silenced. (Liu et al 2008). Regarding the present MIE, PPARα antagonists such as GW6471 stabilize the binding of co-repressors to the PPARα signaling complex suppressing nuclear signaling and thus downstream transactivation-transcription of PPARα-regulated genes. Given that PPARα trans-activation induces catabolism of fatty acids, this signaling pathway has been broadly demonstrated to play a key role in energy homeostasis (Kersten 2014, Evans et al 2004, Desvergne and Wahli 1999).

Evidence Supporting this KER

Stabilization of co-repressors to the PPARα signaling complex suppresses nuclear signaling (Xu et al. 2002). Impaired PPARα nuclear signaling has been broadly demonstrated to decrease the transcriptional expression of PPARα-regulated genes (Kersten et al 2014). Thus, the KER for the KE "PPAR alpha co-repressor stabilization, increased" -> the KE "PPARalpha transactivation of gene expression, decreased" received the score of "strong".

Biological Plausibility

The biological plausibility is high given the crystal structure resolved for the bound group of GW6471, the co-repressor SMRT, and PPARα where the ligand binding domain of PPARα was set in the inactive conformation (Xu et al 2002). Additionally, Krogsdam et al (2002) have established dose-response relationships for increasing N-CoR activity with decreased fold induction of PPARα transactivation potential.

Empirical Evidence

Include consideration of temporal concordance here

See supporting evidence in previous bullets. The binding of the co-repressor to the PPARα complex occurs in advance of suppression of PPARα transactivation (Xu et al 2002).

Uncertainties and Inconsistencies

Given the observations that co-repressors can inhibit PPARα nuclear signaling (Xu et al 2002) and downstream transactivation potential (Krogsdam et al 2002), each in a dose-responsive manner, this provides strong evidence for the present KER. It should be noted however that there are a variety of structural elements included in the PPARα nuclear signaling complex, including the action of co-activators (Xu et al 2001), so there is potential for modifiers in the signaling cascade.

Quantitative Understanding of the Linkage

Krogsdam et al (2002) have established dose-response relationships for increasing N-CoR activity with decreased fold induction of PPARα transactivation potential.

Response-response relationship

Unknown.

Time-scale

Rapid Molecular Interactions.

Known modulating factors

Unknown.

Known Feedforward/Feedback loops influencing this KER

Unknown.

References

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Relationship: 1748: Decreased, PPARalpha transactivation of gene expression leads to Fatty Acid Beta Oxidation, Decreased

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonist binding to PPARα leading to body-weight loss	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	High	NCBI
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

The relationships described herein have been primarily established in human and rodent models although the processes are fundamental in biology and likely broadly conserved.

Key Event Relationship Description

PPARα acts as a positive transcriptional regulator for many of the genes involved in peroxisomal and mitochondrial fatty acid beta oxidation as well as genes involved in the pre- and post-processing of fatty acids in peroxisomal pathways (Desvergne and Wahili 1999, Kersten 2014). Inhibition of PPARα transactivation (KE1) results in decreased transcriptional expression for genes that catalyze the peroxisomal and mitochondrial fatty

acid beta oxidation pathways (Desvergne and Wahili 1999, Kersten 2014, Dreyer et al. 1992, Lazarow 1978) by inhibiting expression of the enzymes involved in fatty acid metabolism. The processes involved in both peroxisomal and mitochondrial fatty acid beta-oxidation, are well described in the literature including good coverage of the gene products that catalyze the metabolic reactions (Kersten 2014) with reasonable characterization of metabolic flux (Mannaerts and Van Veldhoven 1993, Desvergne and Wahli 1999), thus the WOE scores for KER were in the medium to medium-high range.

Evidence Supporting this KER

PPARα acts as a positive transcriptional regulator for many of the genes involved in both peroxisomal and mitochondrial fatty acid beta oxidation as well as genes involved in the pre- and post-processing of fatty acids in both pathways (Desvergne and Wahili 1999, Kersten 2014), hence the KER for the KE, "decreased PPARα transactivation of gene expression" -> the KE "decreased fatty acid beta oxidation" received the score of "strong". Peroxisomal fatty acid beta oxidation reactions shorten very long chain fatty acids from dietary sources releasing acetyl-CoA subunits (a primary metabolic fuel source) and shortened-chain fatty acids that can subsequently be catabolized in the downstream KE, "mitochondrial fatty acid beta-oxidation" (as reviewed in Kersten et al. 2014 and Desvergne and Wahli 1999). Mitochondrial processing of fatty acids involves: (1) Import of short, medium and long chain fatty acids (<C20) acyl-CoAs into the mitochondrial, (2) beta-oxidation catalyzed by the length-specific acyl-CoA hydrogenases, (3) release acetyl-CoAs from the hydrocarbon chain and, (4) conversion of unsaturated and 2-methylated acetyl-CoAs into intermediates for beta-oxidation (Brandt et al 1998, Gulick et al 1994, Mascaro et al 1998, Sanderson et al 2008, Aoyama et al 1998, Kersten et al 2014).

Biological Plausibility

Biological plausibility of this KER is strong given the supporting relationships cited in the literature described in the previous bullets above.

Empirical Evidence

PPARα knock out nullifies downstream expression of transcripts for genes involved in both peroxisomal and mitochondrial beta-oxidation of fatty acids (Kersten et al 2014).

Uncertainties and Inconsistencies

The KER relationship between the KE, "decreased PPARα transactivation of gene expression" and the KE, "decreased fatty acid beta oxidation" is well supported by the literature (see references above). Few uncertainties remain, and few inconsistencies have been reported.

Quantitative Understanding of the Linkage

A large body of research demonstrated that PPARα nuclear signaling directly controls transcriptional expression for genes catalyzing peroxisomal beta-oxidation of very long chain fatty acids (>20C), mitochondrial beta-oxidation of short, medium and long chain fatty acids (<20C), and ketogenesis (as reviewed in Kersten 2014, Evans et al 2004, Desvergne and Wahli 1999, Sanderson et al 2010). The majority of the research described

in these reviews was established using gene knock outs, so there is not much dose-response information available describing the KE, “decreased PPARα transactivation of gene expression” -> the KE, “decreased fatty acid beta oxidation”.

Response-response relationship

Unknown.

Time-scale

Rapid Molecular Interactions.

Known modulating factors

Unknown.

Known Feedforward/Feedback loops influencing this KER

Unknown.

References

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Relationship: 880: Decreased, PPARalpha transactivation of gene expression leads to Decreased, Ketogenesis (production of ketone bodies)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonist binding to PPARα leading to body-weight loss	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Mus musculus	Mus musculus	High	NCBI
Homo sapiens	Homo sapiens	High	NCBI
Rattus rattus	Rattus rattus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	Moderate

Sex Applicability

Sex	Evidence
Male	Moderate
Female	Moderate

Evidence provided for human in Cahill (2006), Owen et al (2005) and Williamson et al (1962). Evidence for mouse provided in Kersten et al (1999). Comparative investigations of ketone body formation comparing human and mouse is not well established relative to fatty-acid oxidation comparisons.

Key Event Relationship Description

PPARα is a transcriptional regulator for a variety of genes that facilitate systemic energy homeostasis (Kersten 2014, Evans et al 2004, Desvergne and Wahli 1999). The KE, "decreased PPARα transactivation of gene expression" results in the KE, "decreased transcriptional expression for genes that catalyze ketogenesis" (Cahil 2006, Kersten et al. 2014, Sengupta et al. 2010, Desvergne and Wahli 1999) by inhibiting expression of the enzymes involved in ketogenesis. Enzyme description (Kersten 2014, Sengupta et al. 2010) and metabolic flux examinations (Sengupta et al. 2010) additionally providing fairly robust characterization in support of the KER. Ketogenesis is critical to supporting general systemic energy homeostasis in fasting events (Cahill 2006, Evans et al 2004, Sengupta et al 2010), thus KE4 becomes important after short term energy stores (glycogen) become limited (Muoio et al 2002). Le May, et al (2000) have shown decreased ketogenesis in livers of PPAR null mice linked to impaired mitochondrial hydroxymethylglutaryl-CoA synthase (Hmgcs) gene expression.

Evidence Supporting this KER

Inhibition of PPARα signaling has been demonstrated to inhibit transcriptional expression of genes that catalyze ketogenesis as well as ketone body production (Badman et al 2007, Potthoff 2009, Sengupta 2010) affecting circulating levels of ketone bodies for systemic use. Kersten et al (1999) demonstrated that PPARα is induced in fasted mice mobilizing the oxidation of fatty acids for energy production. In that study, PPARα-null mice did not actively induce fatty acid oxidation or ketogenesis leaving the mice unable to meet energy demands during fasting and leading to hypoglycemia, hyperlipidemia, hypoketonemia and fatty liver. Upstream metabolic events, such as the KEs "peroxisomal fatty acid beta oxidation" and "mitochondrial fatty acid beta oxidation" play a key role in producing substrates for ketogenesis. Although the connection between the KE, "decreased PPARα transactivation of gene expression" -> the KE, "decreased ketogenesis" is well established given the literature cited above, the dependency on the KEs "peroxisomal fatty acid beta oxidation" and "mitochondrial fatty acid beta oxidation" for substrate availability can affect the KE, "decreased ketogenesis" in addition to the influence of the up-stream KE, "decreased PPARα transactivation of gene expression, therefore we scored the KER for the KE, "decreased PPARα transactivation of gene expression" -> the KE, "Ketogenesis (production of ketone bodies)" as "moderate".

Biological Plausibility

Biological plausibility of this KER is strong given the supporting relationships cited in the literature described in the description above.

Empirical Evidence

Include consideration of temporal concordance here

Given that inhibition of PPARα transactivation results in downstream inhibition of transcriptional expression for the genes that catalyze ketogenesis, as well as ketone body production (Badman et al 2007, Potthoff 2009, Sengupta 2010), that KE occurs prior to the KE of decreased ketogenesis.

Uncertainties and Inconsistencies

A critical data gap regarding this AOP is an absence of studies that have investigated the effects null mutants for ketogenesis on the physiology and individual performance during long term starvation relative to wild type individuals.

Quantitative Understanding of the Linkage

Enzyme description (Kersten 2014, Sengupta et al. 2010) and metabolic flux examinations (Sengupta et al. 2010) additionally providing fairly robust characterization in support for the KE of decreased ketogenesis. Little dose-response information is available regarding decreased transcriptional expression of genes involved in ketogenesis and ketone body production.

Response-response relationship

Ketogenesis is more prevalent in fasted state.

Time-scale

A period of fasting such that available glucose is consumed is usually a pre-requisite for increased ketogenesis.

Known modulating factors

Availability of alternative energy substrates may chance the dynamics of this KER.

Known Feedforward/Feedback loops influencing this KER

Ketogenesis diminishes after transition from a fasted state to a fed state.

References

Badman MK, Pissios P, Kennedy AR, Koukos G, Flier JS, Maratos-Flier E: Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell metabolism* 2007, 5(6):426-437.

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Sengupta S, Peterson TR, Laplante M, Oh S, Sabatini DM: mTORC1 controls fasting-induced ketogenesis and its modulation by ageing. *Nature* 2010, 468(7327):1100-1104.

Williamson DH, Mellanby J, Krebs HA: Enzymic determination of d(−)-β-hydroxybutyric acid and acetoacetic acid in blood. *Biochem J* 1962, 82(1):90-96.

Relationship: 1749: Fatty Acid Beta Oxidation, Decreased leads to Decreased, Ketogenesis (production of ketone bodies)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonist binding to PPARα leading to body-weight loss	adjacent	Moderate	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	Moderate	NCBI
human	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Male	Moderate
Female	Moderate

The relationships described herein have been primarily established in human and rodent models. Comparative investigations of ketone body formation comparing human and mouse is not well established relative to fatty-acid oxidation comparisons.

Key Event Relationship Description

Peroxisomes participate in a variety of lipid metabolic pathways including the beta-oxidation of very long-straight chain (<20 C in length) or branched –chain acyl-CoAs (Lazarow 1978, Kersten 2014). The peroxisomal beta-oxidation pathway is not directly coupled to the electron transport chain and oxidative phosporylation, therefore the first oxidation reaction loses energy to heat (H2O2 production) while in the second step, energy is captured in the metabolically accessible form of high-energy electrons in NADH (Mannaerts and Van Veldhoven 1993, Desvergne and Wahli 1999). The peroxisomal beta-oxidation pathway provides fatty acid chain shortening where two carbons are removed in each round of oxidation in the form of acetyl-CoA (Desvergne and Wahli 1999). The shortened chain fatty acids (<20C) can then be transported to the mitochondria to undergo mitochondrial beta-oxidation for complete metabolism of the carbon substrate for cellular energy production (Desvergne and Wahli 1999). Mitochondrial fatty acid beta oxidation catabolizes short, medium and long chain fatty acids (<C20) into acetyl-CoA and ATP. The production of acetyl-CoA monomers is important as they serve as fundamental units for metabolic energy production (ATP) via the citric acid cycle followed by electron-transport chain mediated oxidative phosphorylation (Nelson and Cox, 2000A). Acetyl-CoA is also a fundamental units of energy storage via gluconeogenesis (Nelson and Cox, 2000B) and lipogenesis (Nelson and Cox, 2000C). The liver plays a key role in processing the fundamental energy substrate, acetyl-CoA, into metabolic currencies that contribute to the systemic cellular energy needs of the whole organism. The liver represents a key organ involved in systemic energy distribution given its ability to synthesize glucose (an ability

shared only with the kidney, Gerich et al 2001) as well as its exclusive role in the generation of ketone bodies (Cahill 2006, Sengupta et al 2010, Kersten 2014). This is especially important for the metabolic energy needs of the brain which can only use glucose and the ketone body, β-hydroxybutyrate for cellular energy production (Cahill 2006, Owen 2005, Kersten 2014). Therefore, the KE, "ketogenesis (production of ketone bodies)" is critical to supporting general systemic energy homeostasis in fasting events (Cahill 2006, Evans et al 2004, Sengupta et al 2010).

Evidence Supporting this KER

The KER scores for weight of evidence for the KE, "fatty acid beta oxidation" -> the KE, "ketogenesis (production of ketone bodies)" was considered "strong" given that the former serves as a primary source of substrate for the latter (Badman et al. 2007, Potthoff et al. 2009). Interference with ketogenesis, for example by PPARα inhibition, has been demonstrated to inhibit β-hydroxybutyrate production (measured in serum) during fasting events in mice (Badman et al 2007, Potthoff 2009, Sengupta et al 2010). The quantitative understanding score for this KER was considered "moderate" given that weight of evidence for the individual KEs was robust and the results in a study by Badman et al (2007) indicating that metabolism of fatty acid substrates (measured as liver triglycerides) that would otherwise contribute to β-hydroxybutyrate production was additionally inhibited under PPARα knockout. Further, Le May et al (2000) have shown decreased ketogenesis in livers of PPAR null mice linked to impaired mitochondrial hydroxymethylglutaryl-CoA synthase (Hmgcs) gene expression.

Biological Plausibility

Biological plausibility of this KER is strong given the supporting relationships cited in the literature described in the previous bullets above.

Empirical Evidence

As described in the previous sections, there is a fundamental linkage between KEs given that the KE, "fatty acid beta oxidation" produces raw materials that are used in the KE, "ketogenesis (production of ketone bodies)". It is less clear how essential the former is to a sustainable throughput of the latter especially given that the latter can utilize substrates that can be produced by various other cellular energy processing pathways in addition to mitochondrial fatty acid beta oxidation.

Uncertainties and Inconsistencies

Additional investigations tracing substrate processing, specifically from sources resulting from the KE, "fatty acid beta oxidation" under control as well as starvation conditions would supplement current understanding of the connections between the KE, "fatty acid beta oxidation" and the KE, "ketogenesis (production of ketone bodies)".

Quantitative Understanding of the Linkage

As discussed in the previous sections, the degree to which the, KE "fatty acid beta oxidation" affects the KE, "ketogenesis (production of ketone bodies)" is not well described, neither are modulators of the response-response relationships. Certainly, the pathways are interrelated and connected by PPARα as the master regulator of each process,

so additional modulators related to resource availability and cellular signaling require exploration. We are not currently aware of any models available to extrapolate results among KEs.

Response-response relationship

Unknown.

Time-scale

Unknown.

Known modulating factors

Availability of alternative energy substrates may chance the dynamics of this KER.

Known Feedforward/Feedback loops influencing this KER

Ketogenesis diminishes after transition from a fasted state to a fed state.

References

Cahill 2006, Evans et al 2004, Sengupta et al 2010), thus KE4 becomes important after short term energy stores (glycogen) become limited (Muoio et al 2002). Le May, et al (2000)

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Sengupta S, Peterson TR, Laplante M, Oh S, Sabatini DM: mTORC1 controls fasting-induced ketogenesis and its modulation by ageing. Nature 2010, 468(7327):1100-1104.

Relationship: 883: Decreased, Ketogenesis (production of ketone bodies) leads to Not Increased, Circulating Ketone Bodies

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonist binding to PPARα leading to body-weight loss	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	Moderate

Sex Applicability

Sex	Evidence
Male	Moderate
Female	Moderate

The relationships described herein have been primarily established in human and rodent models.

Key Event Relationship Description

The KE, “ketogenesis (production of ketone bodies)” is critical to supporting general systemic energy homeostasis in fasting events (Cahill 2006, Evans et al 2004, Sengupta et

al 2010). Interference with ketogenesis, for example by PPARα inhibition, has been demonstrated to inhibit β-hydroxybutyrate production (measured in serum) during fasting events in mice (Badman et al 2007, Potthoff 2009, Sengupta et al 2010). The Badman et al (2007) study indicated that metabolism of fatty acid substrates (measured as liver triglycerides) that would otherwise contribute to β-hydroxybutyrate production was additionally inhibited under PPARα knockout.

In a fasting state, humans transition from the use of exogenous glucose to glucose derived from glycogen within 4 hours with a steadily increasing proportion of glucose usage that is derived from gluconeogenesis up to 2 days (Cahill 2006). Beyond 2 days of fasting, ketone body production (β-hydroxybutyrate) increasingly supports the energy demands of the brain (Cahill 2006).

Physiological studies of the progression of human starvation have identified that the preferred metabolic fuel is glucose in the fed state and progressing through two days of fasting, afterward ketone bodies become increasingly important for meeting energy demands (Cahill 2006, Owen et al 2005). Substrates derived from carbohydrates, fats and protein can contribute to gluconeogenesis (Cahill 2006, Gerich et al 2001) whereas substrates derived from fatty acids are the primary contributors to ketogenesis (Desvergne and Wahli 1999). Mobilization of fatty acids as a metabolic fuel source increase dramatically during fasting to support both gluconeogenesis and ketogenesis (Evans et al 2004). Cahill (2006) and colleagues have demonstrated the importance of ketone body production, especially β-hydroxybutyrate, for maintaining energy homeostasis during starvation. β-hydroxybutyrate serves as an alternative substrate to glucose for providing energy to the brain in the starvation state, providing ATP at higher efficiency relative to the glucose substrate (Cahill 2006). Interference with ketogenesis, for example by PPARα inhibition, has been demonstrated to inhibit β-hydroxybutyrate production (measured in serum) during fasting events in mice (Badman et al 2007, Potthoff 2009, Sengupta et al 2010) and cause hypoketonemia (Muoio et al 2002). The Badman et al (2007) study indicated that metabolism of fatty acid substrates (measured as liver triglycerides) that would otherwise contribute to β-hydroxybutyrate production was inhibited under PPARα knockout. Increased concentrations of circulating ketone bodies is indicative of potential metabolic fuel deficits in fasting animals (Cahill 2006), and a lack of increase in circulating ketone bodies during fasting, especially in conjunction with elevated blood triglycerides, indicates impaired ketogenesis and potentially impaired bioenergetic potential.

A potential implication of decreased ketone body production is stress on cardiac function given that energy-stressed heart tissue shifts reliance away from fatty acids toward ketone bodies (β-hydroxybutyrate) to fuel production of the ATP needed to maintain the heart's mechanical function (Aubert et al 2016). Related to this observation, PPARα-knockout mice reached exhaustion sooner than wild types in an exercise challenge which corresponded with significantly decreased β-hydroxybutyrate in serum indicating hypoketonemia in PPARα-knockout mice versus wild types (Muoio et al 2002). Overall, diminished PPARα function, especially in combination with fasting /diminished nutrition and/or excessive exercise may contribute to impaired maintenance on systemic energy budget.

Evidence Supporting this KER

As described in the section above, the KER for the KE, "decreased ketogenesis (production of ketone bodies)" -> the KE, "no increase of circulating ketone bodies" is well supported and received a "strong" weight of evidence score given that inhibition of decreased

ketogenesis was demonstrated to reduce circulating ketone body concentrations under fasting conditions, but not relative to fed animals (Sengupta et al. 2010, Badman et al. 2007). The quantitative understanding was scored as "moderate" given that, although there is strong literature support of the quantitative relationships between the KE, "ketogenesis (production of ketone bodies)" and the KE, "no increase of circulating ketone bodies" under starvation events, there is less knowledge available regarding the specific inhibition of the PPARalpha signaling pathway as the source of "starvation" or depletion of available fatty acids as the starting stock for ketogenesis.

Biological Plausibility

Biological plausibility of this KER is strong given the supporting relationships cited in the literature described in the previous bullets above.

Empirical Evidence

Include consideration of temporal concordance here

As described in the bullets above, there is fairly robust empirical support for this KER, including temporal concordance associated with starvation events.

Uncertainties and Inconsistencies

The data is fairly robust. Additional specific systems level investigations with PPARalpha signaling knockouts would be useful for understanding non-starvation related ketogenic processes.

Quantitative Understanding of the Linkage

The results presented in the references cited above provide many of the quantitative relationships among KEs. Multiple signaling pathways are involved in the starvation response, so additional response-response relationships are likely to play a role in the systemic response as well as interaction with the KE, "decreased ketogenesis (production of ketone bodies)" and the KE, "no increase of circulating ketone bodies". We are not currently aware of any models available to extrapolate results among KEs.

Response-response relationship

Unknown.

Time-scale

Unknown.

Known modulating factors

Availability of alternative energy substrates may chance the dynamics of this KER.

Known Feedforward/Feedback loops influencing this KER

Ketogenesis diminishes after transition from a fasted state to a fed state.

References

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Sengupta S, Peterson TR, Laplante M, Oh S, Sabatini DM: mTORC1 controls fasting-induced ketogenesis and its modulation by ageing. *Nature* 2010, 468(7327):1100-1104.

Relationship: 885: Increased, Catabolism of Muscle Protein leads to Decreased, Body Weight

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonist binding to PPARα leading to body-weight loss	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	High
Juvenile	High

Sex Applicability

Sex	Evidence
Male	High
Female	High

This KER is generally applicable to animal systems.

Key Event Relationship Description

After two to three days of fasting in humans, dietary glucose has been long-since expended and contribution to blood glucose from glycogen metabolism is reduced to zero (Cahill 2006). At this point, about two fifths of fatty acid metabolism in the whole body is dedicated to hepatic ketogenesis, largely in support of the energy demands of the brain, however the brain is still significantly supported by glucose derived from gluconeogenesis (Cahill 2006). PPARα knockout mice that were either fasted or exercised to exhaustion had diminished capacity for maintaining energetic substrates in serum (glucose and lactate) while showing diminished capacity for fatty acid oxidation (serum nonesterified fatty acids) and decreased ketogenesis resulting in hypoketonemia (decreased serum β-hydroxybutyrate) relative to wild types (Muoio et al 2002). As fatty acid stores are depleted or become unusable (as in the PPARα knockout condition described above), gluconeogenesis from other substrates becomes increasingly important including muscle protein catabolism *in situ* for supporting muscle function as well as releasing glutamine (Marliss et al 1971) and alanine (Felig et al 1970A) which can be recycled to glucose by gluconeogenesis in the kidney (Goodman et al 1966, Kashiwaya et al 1994, Cahill 2006). Renal gluconeogenesis from glutamine and alanine supports two fifths of new glucose production while the remaining three fifths is produced in liver from, (a) alanine derived from muscle and nonhepatic splanchnic bed, (b) recycled lactate and pyruvate from red blood cells and renal medulla, (c) glycerol from adipose lipolysis and (d) small amounts of β-hydroxybutyrate are recycled to glucose (Cahill 2006). Blood concentrations of alanine exert control over hepatic glucose production and thus also represent a diagnostic

of alanine contribution from muscle to support gluconeogenesis (Cahill 2006, Felig et al 1970B). In prolonged starvation events, the catabolism of muscle protein (KE6) for gluconeogenesis in order to support systemic energy needs results in loss of muscle mass which contributes to loss of overall body weight. Although it has not yet been investigated experimentally, it is plausible based on the results described above for Muoio et al (2002) that diminished PPARα signaling capacity could exacerbate muscle wasting in long-term fasting and/or malnutrition events.

Dynamic energy budget theory has provided useful insights on how organisms take up, assimilate and then allocate energy to various fundamental biological processes including maintenance, growth, development and reproduction (Nisbet et al 2000). Regarding energy allocation, somatic maintenance (maintaining homeostasis) must first be met before then growth may occur, followed by maturation and then finally, surplus energy is dedicated to reproduction (Nisbet et al 2000). If somatic maintenance cannot be sustained, energy substrates must be generated using standing biomass from non-essential organs, such as skeletal muscle, to maintain homeostasis ultimately leading to the ultimate AO of weight loss (Cahill 2006). As an example of the importance of energy allocation to ecological fitness, a review by Martin et al (1987) demonstrated that energy availability (availability of food) was the predominant limiting factor in reproductive success and survival for both young and parents in a broad life history review for bird species. This is a likely scenario for many organisms.

Evidence Supporting this KER

Given the evidence described above, the KER for the KE, "increased, catabolism of muscle protein" -> the AO, "decreased body weight", received a "strong" weight of evidence score given that catabolism and thus loss of muscle mass to sustain systemic energy requirements has been broadly documented as a source of body weight loss (Cahill 2006).

Biological Plausibility

Biological plausibility of this KER is strong given the supporting relationships cited in the literature described in the previous bullets above.

Empirical Evidence

Include consideration of temporal concordance here

During starvation, the loss of muscle mass to sustain systemic energy requirements has been broadly documented as a source of body weight loss (Cahill 2006). Body weight loss can occur via component weight loss from nearly all organ systems. In starvation conditions, loss of muscle mass is essentially always connected to overall body-weight loss. Regarding temporal concordance, the process is reversed after feeding resumes.

Uncertainties and Inconsistencies

No uncertainties presented.

Quantitative Understanding of the Linkage

Specific thresholds for translating the KE, "increased, catabolism of muscle protein" -> the AO, "decreased body weight" are species specific. Models for investigating these

relationships are available including dynamic energy budget models (Nisbet et al 2000) as well as variety of detailed caloric models for humans.

Response-response relationship

Unknown.

Time-scale

The timescale is dependent on availability of alternative energy reserves including glycogen and fatty acids to support systemic energy metabolism.

Known modulating factors

Availability of alternative energy substrates may chance the dynamics of this KER.

Known Feedforward/Feedback loops influencing this KER

Ketogenesis diminishes after transition from a fasted state to a fed state.

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List of Non Adjacent Key Event Relationships

Relationship: 1023: Binding of antagonist, PPAR alpha leads to stabilization, PPAR alpha co-repressor

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonist binding to PPARα leading to body-weight loss	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	Not Specified

Sex Applicability

Sex	Evidence
Male	High
Female	High

The majority of the studies cited herein provide evidence for human and rat, however much of the signaling architecture is also present in yeast (Krogsdam et al 2002).

Key Event Relationship Description

Binding of molecules to peroxisome proliferator-activated receptor α (PPARα) can cause either agonistic or antagonistic signaling depending on molecular structure (Xu et al 2001, Xu et al 2002). Certain molecules that can bind to the PPARα ligand binding domain have been observed to cause conformational changes that induce increased affinity to co-repressors which decrease PPARα nuclear signaling (Xu et al 2002). The transcription co-repressors, silencing mediator for retinoid and thyroid hormone receptors (SMRT) and

nuclear receptor co-repressor (N-CoR) have been observed to compete with transcriptional co-activators for binding to nuclear receptors (including PPARα) thus suppressing nuclear signaling activity (Nagy et al 1999, Xu et al 2002). Regarding the present MIE, PPARα antagonists such as GW6471 which leads to the KE where increased binding and stabilization of the co-repressors to the PPARα signaling complex suppressing nuclear signaling.

Evidence Supporting this KER

As a demonstration of the connection between this MIE and the KE, it has been demonstrated that antagonists such as GW6471 bound to PPARα can recruit and stabilize the binding of co-repressors to the PPARα signaling complex suppressing nuclear signaling (Xu et al. 2002). This relationship has been demonstrated using x-ray crystallography and a variety of additional binding and signaling assays (Xu et al 2002). Additionally, a natural human variant (V227A) in the hinge region of PPARα has been demonstrated to stabilize PPARα/N-CoR interactions resulting in inhibited transactivation of downstream genes in hepatic cells (Liu et al 2008). Therefore, this KER received the score of "strong".

Biological Plausibility

The biological plausibility is high given the crystal structure resolved for the bound group of GW6471, the co-repressor SMRT, and PPARα where the ligand binding domain of PPARα was set in the inactive conformation (Xu et al 2002).

Empirical Evidence

Include consideration of temporal concordance here

The inclusion of GW6471 was observed to recruit binding of the co-repressors SMRT and NCOR to PPARα in a positive dose-responsive manner (Xu et al 2002). Additionally, the application of the antagonist GW6471 was observed to displace the PPARα agonist GW409544 thus reducing PPARα signaling (Xu et al 2002) .The MIE occurs in advance of co-repressor recruitment and changes in PPARα signaling (Xu et al 2002).

Uncertainties and Inconsistencies

Regarding the present MIE, GW6471 has highly specific binding to the SMRT and N-CoR binding domains (Nagy et al 1999, Xu et al 2002). The degree to which other chemicals cause PPARα antagonism by this specific MIE needs to be explored. For example, Wilbanks et al. (2014) and Gust et al (2015) demonstrated inhibition of human PPARα nuclear signaling in *in vitro* nuclear signaling bioassays in response to 2,4-dinitrotoluene(2,4-DNT) and 2-amino-4,6-dinitrotoluene (2A-DNT), respectively. However, it is unknown if this response was manifested through the co-repressor binding stabilization that was identified in (Xu et al 2002).

Quantitative Understanding of the Linkage

A concentration-response curve has been developed for GW6471 recruiting binding of the SMRT and N-CoR co-repressors to the PPARα complex (Xu et al 2002).

Response-response relationship

Unknown.

Time-scale

Rapid Molecular Interactions.

Known modulating factors

Unknown.

Known Feedforward/Feedback loops influencing this KER

Unknown.

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Relationship: 884: Not Increased, Circulating Ketone Bodies leads to Increased, Catabolism of Muscle Protein

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Antagonist binding to PPARα leading to	adjacent	Moderate	Low

body-weight loss

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adults	Moderate

Sex Applicability

Sex	Evidence
Male	Moderate
Female	Moderate

The relationships described herein have been primarily established in human and rodent models.

Key Event Relationship Description

A fundamental process in all biological systems is the production of metabolic fuel for use in meeting the energy demands of cells and the systemic energy needs of multi-cellular organisms. Physiological studies of the progression of human starvation have identified that the preferred metabolic fuel is glucose in the fed state and progressing through two days of fasting, afterward ketone bodies become increasingly important for meeting energy demands (Cahill 2006, Owen et al 2005). Substrates derived from carbohydrates, fats and protein can contribute to gluconeogenesis (Cahill 2006, Gerich et al 2001) whereas substrates derived from fatty acids are the primary contributors to ketogenesis (KE5, Desvergne and Wahli 1999). Mobilization of fatty acids as a metabolic fuel source increase dramatically during fasting to support both gluconeogenesis and ketogenesis (Evans et al 2004). Cahill (2006) and colleagues have demonstrated the importance of ketone body production, especially β-hydroxybutyrate, for maintaining energy homeostasis during starvation. β-hydroxybutyrate serves as an alternative substrate to glucose for providing energy to the brain in the starvation state, providing ATP at higher efficiency relative to the glucose substrate (Cahill 2006). Interference with ketogenesis, for example by PPARα inhibition, has been demonstrated to inhibit β-hydroxybutyrate production (measured in serum) during fasting events in mice (Badman et al 2007, Potthoff 2009, Sengupta et al 2010). The Badman et al (2007) study indicated that metabolism of fatty acid substrates

(measured as liver triglycerides) that would otherwise contribute to β-hydroxybutyrate production was inhibited under PPARα knockout. Increased concentrations of circulating ketone bodies is indicative of potential metabolic fuel deficits in fasting animals (Cahill 2006), and a lack of increase in circulating ketone bodies during fasting, especially in conjunction with elevated blood triglycerides, indicates impaired ketogenesis and potentially impaired bioenergetic potential.

After two to three days of fasting in humans, dietary glucose has been long-since expended and contribution to blood glucose from glycogen metabolism is reduced to zero (Cahill 2006). At this point, about two fifths of fatty acid metabolism in the whole body is dedicated to hepatic ketogenesis, largely in support of the energy demands of the brain, however the brain is still significantly supported by glucose derived from gluconeogenesis (Cahill 2006). PPARα knockout mice that were either fasted or exercised to exhaustion had diminished capacity for maintaining energetic substrates in serum (glucose and lactate) while showing diminished capacity for fatty acid oxidation (serum nonesterified fatty acids) and decreased ketogenesis resulting in hypoketonemia (decreased serum β-hydroxybutyrate) relative to wild types (Muoio et al 2002). As fatty acid stores are depleted or become unusable (as in the PPARα knockout condition described above), gluconeogenesis from other substrates becomes increasingly important including muscle protein catabolism *in situ* for supporting muscle function as well as releasing glutamine (Marliss et al 1971) and alanine (Felig et al 1970A) which can be recycled to glucose by gluconeogenesis in the kidney (Goodman et al 1966, Kashiwaya et al 1994, Cahill 2006). Renal gluconeogenesis from glutamine and alanine supports two fifths of new glucose production while the remaining three fifths is produced in liver from, (a) alanine derived from muscle and nonhepatic splanchnic bed, (b) recycled lactate and pyruvate from red blood cells and renal medulla, (c) glycerol from adipose lipolysis and (d) small amounts of β-hydroxybutyrate are recycled to glucose (Cahill 2006). Blood concentrations of alanine exert control over hepatic glucose production and thus also represent a diagnostic of alanine contribution from muscle to support gluconeogenesis (Cahill 2006, Felig et al 1970B). In prolonged starvation events, the catabolism of muscle protein (KE6) for gluconeogenesis in order to support systemic energy needs results in loss of muscle mass which contributes to loss of overall body weight. Although it has not yet been investigated experimentally, it is plausible based on the results described above for Muoio et al (2002) that diminished PPARα signaling capacity could exacerbate muscle wasting in long-term fasting and/or malnutrition events.

Evidence Supporting this KER

The scoring for the KER, where the KE, “no increase in circulating ketone bodies” -> the KE, increased, catabolism of muscle protein” reflected the observation that amino acid recycling to glucose via renal gluconeogenesis (Goodman et al. 1966, Kashiwaya et al. 1994) is a broadly separate process and under separate regulatory control from ketogenesis (Felig et al. 1970B, Sengupta et al. 2010). However, under starvation conditions in conjunction with diminished circulating ketone bodies to serve as fuel for systemic metabolism, an increase in catabolism of muscle protein would be required to support renal gluconeogensis to sustain the systemic energy budget. Therefore, although the KER, for the KE, “no increase in circulating ketone bodies” -> the KE, increased, catabolism of muscle protein” lacks a mechanistic connection, a strong correlative relationship exists

between the KEs regarding energy homeostasis, hence the weight of evidence for the KER was scored as “moderate” however the quantitative understanding was scored as “weak”.

Biological Plausibility

Biological plausibility of this KER is strong given the supporting relationships cited in the literature described in the previous bullets above.

Empirical Evidence

Include consideration of temporal concordance here

As described in the bullets above, there is fairly robust empirical support for this KER, although dedicated assays providing mechanistic / quantitative relationships between amino acid recycling for energy production relative to ketogenesis-based energy production are still needed.

Uncertainties and Inconsistencies

See previous section.

Quantitative Understanding of the Linkage

Although the KER, for the KE, “no increase in circulating ketone bodies” -> the KE, “increased, catabolism of muscle protein” lacks a mechanistic connection, a strong correlative relationship exists between the KEs regarding energy homeostasis. Discovering response-response relationships regarding this complex signaling network represents a key basic research question related to systemic energy metabolism. We are not currently aware of any models available to extrapolate results among KEs.

Response-response relationship

Unknown.

Time-scale

The timescale is dependent on availability of alternative energy reserves including glycogen and fatty acids.

Known modulating factors

Availability of alternative energy substrates may chance the dynamics of this KER.

Known Feedforward/Feedback loops influencing this KER

Ketogenesis diminishes after transition from a fasted state to a fed state

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