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Feature Review

Mechanisms of hepatic fatty acid oxidation and ketogenesis during fasting

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Fasting is part of many weight management and health-boosting regimens. Fasting causes substantial metabolic adaptations in the liver that include the stimulation of fatty acid oxidation and ketogenesis. The induction of fatty acid oxidation and ketogenesis during fasting is mainly driven by interrelated changes in plasma levels of various hormones and an increase in plasma nonesterified fatty acid (NEFA) levels and is mediated transcriptionally by the peroxisome proliferator-activated receptor (PPAR)α, supported by CREB3L3 (cyclic AMP-responsive element-binding protein 3 like 3). Compared with men, women exhibit higher ketone levels during fasting, likely due to higher NEFA availability, suggesting that the metabolic response to fasting shows sexual dimorphism. Here, we synthesize the current molecular knowledge on the impact of fasting on hepatic fatty acid oxidation and ketogenesis.

Highlights

Metabolic adaptations to starvation promote survival when food is scarce but predispose to obesity and diabetes in today's world of caloric excess.

Fasting describes the willful refrain from eating and becomes starvation when an involuntary or prolonged lack of food intake occurs.

Fasting induces a coordinated transcriptional program in the liver mediated chiefly by peroxisome proliferator-activated receptor α (PPARα) that causes the activation of fatty acid oxidation and ketogenesis.

Data from humanized mouse models suggest the conservation of the key molecular pathways governing the fasting response in the human liver.

Available evidence suggests a sexually dimorphic response to fasting between men and women.

Understanding fasting

Fasting is generally considered as the willful refrain from eating, and is a common practice of spiritual or religious rituals. In addition, many popular weight-loss diets advocate a form of fasting regimen [1]. In medical and physiological terms, fasting describes voluntary abstinence from food but not water, and becomes starvation if the lack of food intake is involuntary or becomes long-lasting. In this review, we use the term fasting as a collective description of complete caloric deprivation.

Recurrent periods of undernutrition and fasting were common throughout human evolution. Consequently, fasting has been a key selective pressure shaping nutrient and energy metabolism [2]. The intricate mechanisms directing nutrient and energy metabolism during fasting allowed our ancestors to survive long periods without food. In the modern world of caloric excess, however, these same mechanisms contribute to the unprecedented growth in obesity and related diseases [3]. While much research is aimed at understanding how human metabolism goes awry in obesity and other metabolic diseases, much less attention is paid to what happens during fasting. Further mechanistic understanding of the metabolic response to fasting could hold the key to the design of effective preventive and therapeutic strategies for overnutrition and its pathological sequelae.

Depending on the amount of body fat, humans can tolerate fasting from a few weeks up to several months. During fasting, fuel utilization in tissues changes from a mixture of carbohydrates and fat to primarily fat [4]. This switch in fuel use is partly driven by the increased release of NEFAs from the adipose tissue into the circulation, leading to the gradual depletion of the adipose tissue stores [5]. Other key features are the enhanced production of glucose and ketone bodies in the liver through the processes of gluconeogenesis and ketogenesis, respectively [4]. The glucose and ketone bodies generated are mainly used by the brain, which unlike other tissues can only use fatty acids for fuel to a limited extent [6]. The liver and adipose tissue thus play a key role in

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the adaptive response to fasting. In a previous review, we addressed the changes in adipose tissue metabolism during fasting [5]. Here, we synthesize the evolving knowledge on the impact of fasting on fatty acid oxidation and ketogenesis in the liver.

The role of the liver during fasting

The liver is the central nutrient-processing organ in the body. It acts as a hub by metabolically connecting various tissues, such as the intestine, adipose tissue, skeletal muscle, heart, and brain. During fasting, the liver is essential to direct the flow of nutrients and produce glucose and ketone bodies. The metabolic response to fasting is characterized by different stages of metabolic adaptation [4,7,8]. In the first stage, which covers the period immediately following the ingestion of a meal, the ingested nutrients are digested, absorbed, and stored. During this stage, the liver produces fatty acids, cholesterol, and bile acids; the latter of which are secreted into the bile ducts and assist with the digestion of dietary triglycerides. In the second stage, which starts once intestinal absorption is completed, glycogen stores in the liver are utilized to maintain blood glucose levels through the process of glycogenolysis [9]. At this stage, the adipose tissue switches from net lipid storage to lipid release, leading to elevated NEFA levels in the blood. In the third stage, the liver glycogen stores are depleted, and glucose synthesis through gluconeogenesis becomes the primary source of plasma glucose, which is accompanied by the breakdown of muscle protein to provide gluconeogenic amino acids. At this stage, the liver already starts producing ketone bodies using NEFAs as a substrate. In the fourth stage, which lasts for several weeks, the production of ketone bodies in the liver is fully activated, largely replacing glucose as fuel for the brain and thereby suppressing the need for gluconeogenesis and advanced muscle proteolysis. At this stage, tissues other than the brain rely almost entirely on fatty acids as fuel [10]. The last stage is triggered by the depletion of the fat stores. As a consequence, protein becomes the primary energy source for the body, reflecting a last resort to produce energy and delay imminent death [4,7,8].

During all stages of fasting, the liver also actively secretes triglycerides. The liver takes up NEFAs from the circulation, converts them into triglycerides, and secretes them packaged in very low-density lipoproteins (VLDL) [11]. The VLDL-triglycerides provide fuel to several tissues, including the heart and skeletal muscle, but are also partly re-stored in the adipose tissue. Beyond the post-prandial stage, all triglycerides in the blood are carried in VLDL.

Metabolic adaptation to fasting

Mitochondrial fatty acid oxidation

One of the key effects of fasting on the liver is the marked activation of fatty acid oxidation and ketogenesis [12,13]. The liver is the only organ able to carry out *de novo* ketogenesis. During fasting, the elevated levels of NEFAs in the blood lead to increased hepatic uptake of fatty acids. Upon entry, the fatty acids are converted into acyl-CoA via acyl-CoA synthetases. The acyl-CoA molecules can follow several different routes. First, they can be esterified to triglycerides and stored in lipid droplets. Consequently, liver triglyceride content increases during fasting [14,15]. Second, the acyl-CoAs can be oxidized in the mitochondria to provide energy. This requires that the CoA group is exchanged for carnitine via CPT1A to form acyl-carnitines, which are transported into the mitochondria via the SLC25A20 transporter [16]. After entry into the mitochondria, the acyl-carnitines are reconverted into acyl-CoAs by CPT2. The acyl-CoAs are sequentially oxidized via the process of β-oxidation, resulting in the production of acetyl-CoA. Acetyl-CoA can be further oxidized to CO_2 and H_2O via the tricarboxylic acid (TCA) cycle, resulting in the production of the high-energy electron carriers NADH and $FADH_2$ [16]. NADH and $FADH_2$ donate their high-energy electrons to the electron transport chain, which ultimately leads to the production of ATP.

Ketogenesis

If the delivery of fatty acids to the liver is high, as occurs during fasting, β-oxidation and the TCA cycle cannot keep up with the supply of fatty acids to the liver. Consequently, acyl-carnitines accumulate locally, which spill over into the bloodstream, leading to an increase in plasma levels of short-chain and long-chain acylcarnitines and a delayed decrease in free carnitine [17–19]. In addition, acetyl-CoA accumulates in the mitochondria, which is converted into the ketone bodies β-hydroxybutyrate, acetoacetate, and acetone via reactions catalyzed by acetyl-CoA acetyltransferase (ACAT)1, mitochondrial HMG-CoA synthase (HMGCS)2, HMG-CoA lyase (HMGCL), and β-hydroxybutyrate dehydrogenase (BDH)1 [20]. Acetoacetate can be converted into β-hydroxybutyrate or vice versa depending on the redox state of the mitochondrial matrix. How ketone bodies are transported out of the mitochondria is not known. The ketone bodies are exported from hepatocytes into the circulation via the transporters SLC16A1 and SLC16A7 [21].

The hepatic production and levels of ketone bodies in the blood are highly dependent on nutritional status. The primary ketone body in the blood is β-hydroxybutyrate, levels of which are about fourfold higher than acetoacetate. The plasma levels of β-hydroxybutyrate increase from about 0.1 mM in the postprandial state to 0.5–1.8 mM after 24 h fasting and can exceed 6 mM after several weeks of fasting [4].

Other (patho)physiological conditions accompanied by elevated ketogenesis include the consumption of a ketogenic diet, prolonged exercise, and diabetic ketoacidosis. A ketogenic diet is characterized by low carbohydrate content. As a result, plasma insulin levels remain low, plasma NEFA levels go up, and most of the energy is derived from fatty acid oxidation [22,23], leading to increased ketone body synthesis in the liver. The most ketogenic dietary components are medium-chain fatty acids. This is because medium-chain fatty acids directly go to the liver rather than the adipose tissue and do not require carnitine for mitochondrial uptake. The strong ketogenic properties of medium-chain fatty acids are illustrated by the markedly higher rate of ketogenesis in perfused mouse liver after infusion of octanoate compared with oleate [24], as well as by the 2.8-fold higher plasma concentration of total ketones after 6 months of daily consumption of 30 g of medium-chain triglycerides compared with high oleic acid triglycerides [25].

Prolonged exercise is another physiological stimulus that can result in ketonemia, especially when combined with fasting [21]. Akin to fasting, prolonged exercise markedly activates adipose tissue lipolysis, which leads to elevated plasma NEFAs and enhanced ketogenesis in the liver. The ketone bodies generated can subsequently be metabolized by exercising muscle to generate ATP.

Diabetic ketoacidosis is a dangerous condition most often observed in patients with type 1 diabetes. It is caused by low insulin levels, leading to excessive ketogenesis in the liver and elevated ketones in the blood, which can reach concentrations as high as 16 mM [26]. Since ketone bodies are acids, they lower the blood pH, hence the term ketoacidosis. Diabetic acidosis is accompanied by severe hyperglycemia and is treated by injection of insulin.

Utilization of ketone bodies

In the fasted state, ketone bodies comprise a major fuel for the brain, heart, and skeletal muscles. Based on the measurement of the levels of ketone bodies entering and leaving the brain, Owen and colleagues estimated that the brain derives up to two thirds of its energy requirements from the oxidation of ketone bodies [10]. This study laid the foundation for the notion that the formation of ketone bodies is a vital evolutionary mechanism aimed at providing an alternative fuel source for the brain besides glucose, thereby sparing amino acids and enhancing survival during

prolonged fasting [4]. The preferential use of ketone bodies by the brain during fasting has since been further quantified and visualized with positron emission tomography scans [27,28].

Besides serving as critical fuel for the brain and other tissues, ketone bodies and in particular β-hydroxybutyrate might also act as signaling molecules and influence numerous cellular processes in a variety of cell types [29]. Several reviews have already addressed this topic [21,30]. It should be noted, though, that a coherent and consistent picture of the specific biological and cellular effects of β-hydroxybutyrate has yet to emerge.

Peroxisomal and microsomal fatty acid oxidation

In addition to oxidizing fatty acids in mitochondria, hepatocytes also oxidize fatty acids in peroxisomes and microsomes. Although quantitatively of lesser importance than mitochondrial fatty acid oxidation, peroxisomal fatty acid oxidation is obligatory for the oxidation of various unusual fatty acids, such as branched and very long-chain fatty acids. Since the TCA cycle is not operational in peroxisomes, fatty acids taken up into peroxisomes cannot be fully oxidized. Studies by Wanders have shown that the branched and very long-chain fatty acids are degraded in peroxisomes to short-chain acyl-CoA [31]. The short-chain acyl-CoAs, such as butyryl-CoA and acetyl-CoA, are subsequently coupled to carnitine and shuttled to the mitochondria, where they are completely oxidized to CO_2 and H_2O [32]. Genetic defects in peroxisomal fatty acid oxidation lead to the accumulation of specific intermediary metabolites, which can be associated with various metabolic and neurological symptoms.

Microsomal fatty acid oxidation refers to the ω-oxidation of fatty acids at the methyl end in the endoplasmic reticulum (ER)/microsomal fraction. It is catalyzed by enzymes of the CYP4A class and leads to the generation of dicarboxylic acids, which are further oxidized in the mitochondria or excreted via the urine.

Circulating factors driving hepatic fatty acid oxidation and ketogenesis during fasting

The induction of hepatic fatty acid oxidation and ketogenesis during fasting is mainly driven by two interrelated factors: changes in plasma levels of various hormones and an increase in plasma NEFA levels [33–35]. Hormones whose levels increase during fasting include cortisol [36–38], growth hormone [36,39], glucagon [36,40,41], adrenaline, and noradrenaline [36,42]. During fasting, low levels of insulin in combination with elevated plasma levels of cortisol, catecholamines, glucagon, and growth hormone promote the hydrolysis of stored triglycerides by stimulating the activity of the lipolytic enzyme adipose triglyceride lipase (ATGL), leading to increased NEFA levels in the blood. An overview of the mechanisms underlying the induction of adipose lipolysis during fasting can be found elsewhere [5]. The stimulation of adipose tissue lipolysis and subsequent elevation of plasma NEFA levels likely for a large part underlie the stimulation of hepatic fatty acid oxidation and ketogenesis by the aforementioned hormones.

Because several metabolic hormones regulate plasma NEFA levels and vice versa, studies performed *in vivo* do not allow the disentanglement of the independent regulatory effects of changes in plasma NEFAs and metabolic hormones. By contrast, *in vitro* studies permit the direct assessment of the effect of metabolic hormones and fatty acids on hepatic fatty acid oxidation and ketogenesis. Collectively, studies in hepatocytes obtained from rats, rabbits, pigs, and humans have shown that glucagon stimulates fatty acid oxidation and ketogenesis in hepatocytes, whereas insulin inhibits these pathways [43–46]. Studies in cultured hepatocytes have similarly shown that increased NEFA levels enhance fatty acid oxidation and ketogenesis, which is mediated by two key mechanisms: (i) increased substrate availability, and (ii) upregulation of various

transporters and enzymes involved in fatty acid oxidation and ketogenesis [47–51]. The latter effects are primarily mediated by specific transcriptional pathways, as discussed further in this review.

Transcriptional pathways driving hepatic fatty acid oxidation and ketogenesis during fasting

Transcriptional regulation

During fasting, changes in plasma levels of various hormones and increased NEFAs trigger the activation of specific transcriptional regulatory pathways, which in turn enhances the enzymatic capacity for fatty acid oxidation and ketogenesis by upregulating the mRNA levels of various enzymes and transporters. An overview of the key transcriptional regulatory pathways activated in the liver during fasting and their target enzymes/transporters is presented (Figure 1, Key figure). Transcriptional pathways that are suppressed during fasting will not be covered here.

Key figure

Schematic integration of the transcriptional networks governing hepatic fatty acid oxidation and ketogenesis during fasting

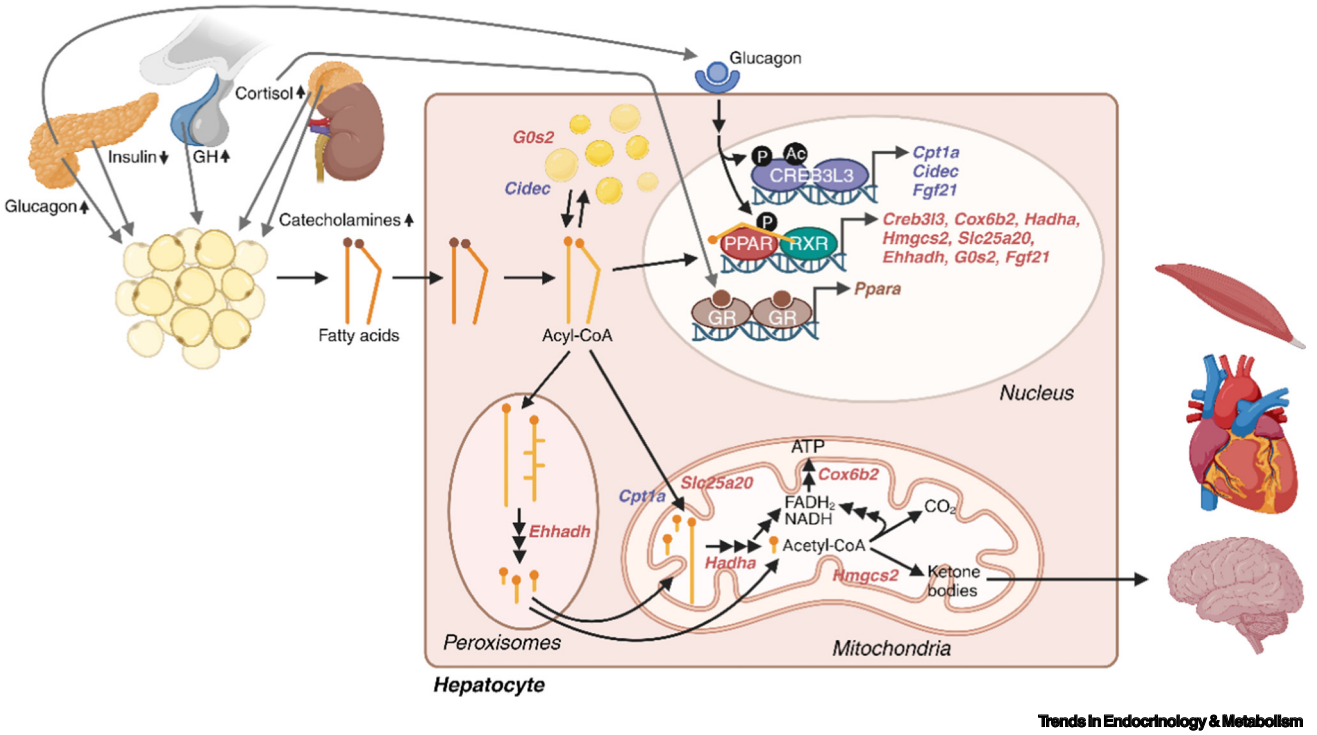

Figure 1. Changes in levels of various hormones stimulate lipolysis in adipose tissue, thereby enhancing the release of NEFAs. The NEFAs are taken up by the liver, where they regulate their own metabolic fate in collaboration with hormonal cues, such as glucagon. Key transcription factors are PPARα (red), CREB3L3 (purple), and GR (brown). Relevant target genes activated during fasting (representative genes depicted in corresponding colors) collectively mediate the transport of fatty acids to mitochondria and peroxisomes, breakdown to acetyl-CoA via β-oxidation, and condensation of acetyl-CoA to ketone bodies through ketogenesis. Subsequently, ketone bodies are secreted into the circulation where they are metabolized by extrahepatic tissues, most notably by the brain. Abbreviations: Ac, acetylation; CREB3L3, cyclic AMP-responsive element-binding protein 3 like 3; GH, growth hormone; GR, glucocorticoid receptor; NEFA, nonesterified fatty acid; P, phosphorylation; PPARα, peroxisome proliferator-activated receptor α. Figure created using BioRender.

PPARα

In addition to serving as metabolic substrates, fatty acids also have a major regulatory role in the activation of fatty acid oxidation and ketogenesis by acting as agonists for the transcription factor PPARα. PPARα is a ligand-activated transcription factor that is expressed at high levels in the liver [52]. Together with PPARβ/δ and PPARγ, it forms a subfamily within the nuclear receptor superfamily. The PPARs have a common mode of action that involves the formation of a heterodimer with the retinoid X receptor (RXR), followed by binding of the PPAR–RXR complex to specific DNA sequences in regulatory regions of target genes [53]. Activation of gene transcription is triggered by the binding of a ligand, followed by the recruitment of coactivator proteins and the loss of corepressor proteins. Ligands for PPARα include fatty acids and fatty acid derivatives such as eicosanoids and oxidized fatty acids, as well as various synthetic molecules collectively referred to as peroxisome proliferators [54]. The latter group includes fibrate drugs used in the treatment of dyslipidemia, as well as plasticizers and forever chemicals belonging to the per- and polyfluoroalkyl substances.

Studies using whole-body or hepatocyte-specific PPARα-deficient mice have unequivocally demonstrated that PPARα is the master regulator of hepatic lipid metabolism during fasting [13,55–58]. As a consequence, fasted PPARα-deficient mice exhibit a host of metabolic abnormalities such as hypoglycemia, hypoketonemia, elevated plasma NEFAs, and steatosis [13,55–58]. Transcriptome profiling of PPARα-deficient livers has shown that these metabolic defects are rooted in the decreased expression of myriads of genes involved in metabolic pathways covering almost all branches of hepatic lipid metabolism [58–61]. Supplementary studies in human or humanized models have shown that much of that regulation by PPARα, although not everything, is copied in human hepatocytes [62–64]. An overview of the fasting-induced changes in the expression of genes involved in mitochondrial fatty acid oxidation and ketogenesis is presented in Figure 2, showing that PPARα upregulates (nearly) every single enzyme involved in these pathways. In addition, PPARα governs the expression of numerous enzymes involved in peroxisomal and microsomal fatty acid oxidation [57–61].

The direct activation of PPARα by fatty acids *in vitro* combined with the pronounced PPARα-dependent activation of genes involved in fatty acid oxidation and ketogenesis by fasting *in vivo* led to the suggestion that the increase in circulating NEFAs mediates the upregulation of PPARα target genes in the liver [13]. In this way, NEFAs govern their own metabolic fate in the liver. In agreement with this notion, in fasted mice, decreased plasma NEFA levels due to adipocyte-specific deficiency of ATGL were accompanied by a profound decrease in the hepatic expression of numerous PPARα target genes involved in fatty acid oxidation and ketogenesis, as well as the abolishment of the fasting-induced increase in plasma ketones [65]. Similarly, reduced plasma NEFA levels due to adipocyte-specific deficiency of the ATGL activator ABHD5 were associated with decreased hepatic expression of several PPARα target genes, including *G0s2*, *Fgf21*, *Acox1*, *Acadm*, and *Acadl* [66]. Conversely, the elevation of plasma NEFAs through stimulation of adipose tissue lipolysis via the β3-adrenergic agonist CL-316243 increased hepatic expression of the PPARα targets *Cpt1b*, *Ehhadh*, and *Pdk4* [67]. It should be noted that treatment with the PPARα ligand Wy14643 was unable to recover the reduced expression of PPARα target genes in adipocyte-specific ABHD5-deficient mice, suggesting a PPARα ligand-independent defect. One possibility is that plasma NEFAs induce PPARα target genes in the liver by upregulating PPARα itself. In line with this notion, changing the NEFAs released during fasting from saturated to unsaturated was associated with hepatic upregulation of *Ppara* and the PPARα target *Fabp1* [13]. Overall, these data indicate that NEFAs are a key signal activating PPARα-dependent pathways in the liver during fasting, although it cannot be definitely established that the NEFAs taken up by the liver directly bind and activate PPARα.

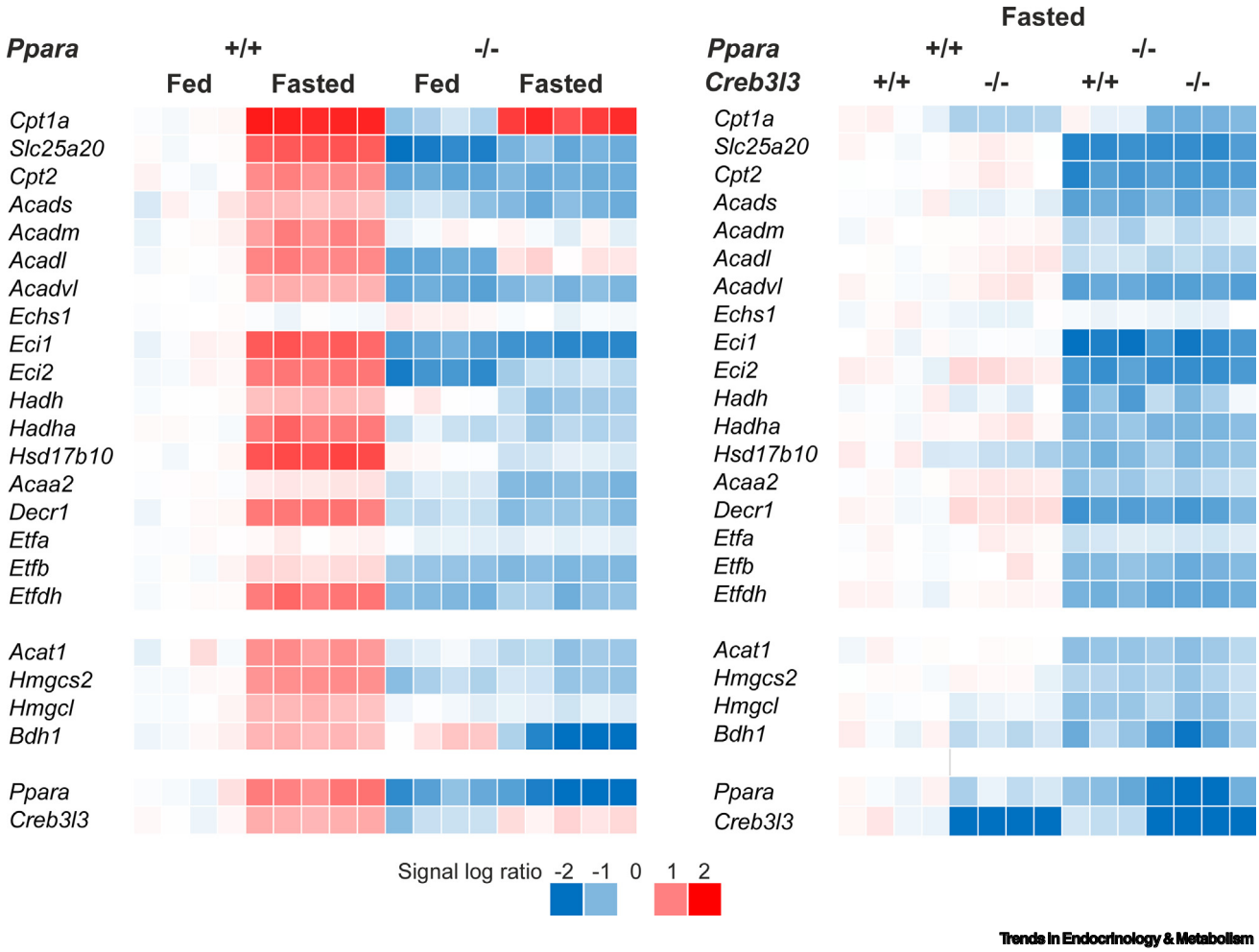

Figure 2. Heatmap of hepatic fatty oxidation and ketogenesis-related genes regulated in mouse liver ablated for PPARα and/or CREB3L3 in response to a 24h or 16h fast [12,60,88]. The ablation of PPARα abrogates the fasting-induced upregulation of all genes, with notable exceptions such as *Cpt1a* or *Acadl* (left panel). Simultaneous ablation of PPARα and CREB3L3 confirms co-regulation of genes *Cpt1a*, *Hsd17b10*, and *Bdh1* and suggests that both transcription factors also regulate each other. Abbreviations: CREB3L3, cyclic AMP-responsive element-binding protein 3 like 3; PPARα, peroxisome proliferator-activated receptor α.

By contrast, variations in plasma NEFA levels due to overexpression or deficiency of ANGPTL4, an inhibitor or activator of extracellular and intracellular lipolysis, respectively, were not associated with any changes in hepatic expression of PPARα target genes or binding of PPARα to their promoter [68]. Other studies indicated that PPARα can be activated by fatty acids produced by lipogenesis and diet-derived fatty acids (the so-called new fat), but not by fatty acids generated by peripheral mobilization (the old fat) [69,70]. Furthermore, studies in the murine heart have indicated that NEFAs coming from plasma may not directly bind and activate PPARα but first need to undergo a cycle of triglyceride synthesis and ATGL-catalyzed intracellular lipolysis [71]. These findings have not been verified in the liver, where ATGL appears to influence PPARα activity independently of ligand-induced activation [72].

As indicated earlier, hepatic PPARα is also activated during fasting by an increase in its expression, which is likely mediated by the glucocorticoid receptor [13,73], as well as by increased coactivation via increased levels of coactivator proteins such as PPARγ coactivator (PGC)-1α

[68]. PGC-1α is the key coactivator involved in the transcriptional control of genes encoding mitochondrial fatty acid oxidation enzymes by PPARα [74,75]. Collectively, it can be concluded that PPARα is activated in the liver during fasting, likely through multiple complementary mechanisms that include increased expression of PPAR and its co-activators, as well as increased activation by fatty acids.

CREB3L3

Another transcription factor that is involved in the regulation of hepatic lipid metabolism during fasting is CREB3L3 (also known as CREBH) [76,77]. CREB3L3 is a bZiP transcription factor that is highly expressed in the liver [78]. It is produced as an ER precursor form and is proteolytically activated in the Golgi at the luminal site and the intramembrane site, releasing the N-terminal portion that functions as a transcriptional activator [78]. In addition to being activated by cAMP- and protein kinase A-dependent phosphorylation, CREB3L3 is activated during fasting by nuclear translocation triggered by adipose tissue-derived fatty acids and via lysine acetylation at residue 294 catalyzed by lysine acetyltransferase P300/CREB-binding protein-associated factor [66,79]. CREB3L3 regulates glucose and lipid metabolism in the liver [80]. Specifically, studies have shown that CREB3L3 stimulates gluconeogenesis [81], glycogenolysis [82], plasma triglyceride clearance [83], and lipid droplet formation [84].

Different lines of evidence suggest an interaction between PPARα- and CREB3L3-mediated gene regulation. First, CREB3L3 is under direct transcriptional control of PPARα via a PPRE located upstream of exon 3 [64,85]. Second, PPARα and CREB3L3 cooperate in the regulation of shared target genes such as *Fgf21* by forming a complex that binds to an integrated CRE-PPAR-responsive element-binding motif in the *Fgf21* gene promoter [86]. The physical interaction between PPARα and CREB3L3 is enhanced by fasting and depends on CREB3L3 acetylation at K294 [79]. Other genes under the dual control of PPARα and CREB3L3 include *Cidec* and *Pck1* [84,87]. However, despite the alleged interaction, the detailed study of the impact of PPARα and CREB3L3 deficiency on hepatic gene expression during fasting revealed very limited overlap [88]. Except for *Cpt1a*, *Hsd17b10*, and *Bdh1*, CREB3L3 does not seem to regulate genes involved in fatty acid oxidation and ketogenesis. Also, no effect was found of CREB3L3 deficiency on in *ex vivo* fatty acid oxidation and fatty acid oxidation genes [89]. In addition, CREB3L3 deficiency was associated with an increase rather than a decrease in fasting plasma β-hydroxybutyrate levels [88,89]. Overall, these data suggest that PPARα and CREB3L3 have distinct functions in the liver during fasting and that CREB3L3 does not regulate fasting ketogenesis [88]. This notion is illustrated by the marked impact of PPARα deficiency, but not CREB3L3 deficiency, on the expression of nearly all genes involved in the mitochondrial fatty acid oxidation and ketogenesis pathways (Figure 2). By contrast, Nakagawa and colleagues observed that CREB3L3 deficiency in the fasted state reduced the expression of many genes involved in fatty acid oxidation, showing synergy with PPARα [76]. The reason for this discrepancy remains unclear.

As mentioned earlier, CREB3L3 and PPARα are important transcriptional regulators of fibroblast growth factor (FGF)21, a hepatokine that is strongly upregulated during fasting in mice [88,90,91]. Several papers have suggested that FGF21 stimulates hepatic ketogenesis [90,91]. However, subsequent studies showed that FGF21 deficiency in mice does not affect ketogenesis [92,93], likely because the FGF21 receptor FGFR1c is absent from the liver. Interestingly, fasting did not significantly increase *FGF21* mRNA in hepatocytes of hepatocyte humanized mice [94]. Furthermore, in humans, plasma FGF21 does not show a notable surge until 7–10 days of fasting [95–97]. Overall, the role of FGF21 in ketogenesis during fasting in mice and humans is controversial.

Forkhead box A2

The forkhead transcription factor forkhead box (FOX)A2 was also proposed to be involved in regulating hepatic fatty acid oxidation and ketogenesis during fasting. Fasting and glucagon were found to promote a shift in FOXA2 localization from the cytoplasm to the nucleus and activate FOXA2 via lysine acetylation [98,99]. Overexpression of FOXA2 induced the expression of genes involved in mitochondrial and peroxisomal β-oxidation and ketogenesis, while FOXA2 deficiency reduced ketone body production by isolated liver mitochondria [98,99]. However, others have questioned the role of FOXA2 in fatty acid oxidation and ketogenesis and have shown that FOXA2 mediates the induction of the hepatic gluconeogenic program via the regulation of genes such as *Pck1* [100,101]. Accordingly, the potential role of FOXA2 in hepatic fatty acid oxidation and ketogenesis requires further study.

Glucocorticoid receptor

A synergy between the glucocorticoid receptor (GR) and PPARα-mediated gene regulation in liver cells has long been recognized [102]. GR directly stimulates PPARα gene transcription by binding to a fasting-induced enhancer within *Ppara* [73,103]. Fluctuations in circulating glucocorticoid levels mediate the circadian regulation of *Ppara* expression in the liver [104]. Just before birth, elevated glucocorticoid levels also stimulate the production of PPARα in the liver of fetal mice to increase the capacity for fat oxidation and thus enabling the newborn mice to harness energy from fat-rich milk [105].

In addition to PPARα being directly regulated by GR at the transcriptional level, GR and PPARα physically interact and are colocalized on chromatin to cooperatively regulate the circadian and fasting-dependent transcription of genes involved in fatty acid oxidation and ketogenesis, such as *Hmgcs2*, *Pdk4*, and *Ehhadh*, possibly via a mechanism in which PPARα facilitates the recruitment of GR to adjacent DNA bindings sites or via tethering binding [106–108]. However, other data argue against this model and suggest that the GR-PPARα crosstalk revolves around the transcriptional induction of PPARα by corticosteroid-activated GR, leading to a gradual increase in enhancer activity of ketogenic genes and subsequent activation of ketogenesis during prolonged fasting [103].

At the functional level, it was reported that the GR agonist dexamethasone inhibits mitochondrial matrix acyl-CoA dehydrogenases and fatty acid β-oxidation in the liver of mice [109], while another study observed that high levels of dexamethasone stimulated ketogenesis [43]. Although interactions between GR and PPARα signaling have been established at the molecular level, clear evidence that hepatic GR influences fatty acid oxidation and ketogenesis in the liver is lacking [110]. Indeed, plasma NEFA and ketone body levels were not significantly altered by hepatocyte-specific deficiency of GR [111]. By contrast, it was recently suggested that ketogenesis might be regulated through GR expressed in resident liver macrophages [112].

Other transcription factors

Another factor that has been suggested to regulate hepatic fatty acid oxidation and ketogenesis during fasting is inositol-requiring enzyme (IRE)1α, which is encoded by the *Ern1* gene [113]. IRE1α is an ER stress sensor that catalyzes the splicing of the mRNA encoding X-box binding protein (XBP)1, leading to the production of the active form called XBP1s. IRE1α and XBP1s were found to be activated by fasting, triggering the transcriptional activation of PPARα and the PPARα-dependent gene expression program [113]. By contrast, hepatocyte-specific XBP1-deficiency was not associated with any changes in plasma β-hydroxybutyrate levels or hepatic expression of PPARα target genes during fasting or on a ketogenic diet [114]. Additional studies are required to verify the existence of a IRE1α-XBP1s-PPARα axis in the liver during fasting.

Another transcription factor implicated in the regulation of fatty acid oxidation and ketogenesis is farnesoid X receptor (FXR). Studies in mice have shown that FXR, consistent with its activation by bile acids, is mainly active in the postprandial state and generally opposes PPARα action and inhibits fatty acid oxidation [115]. By contrast, studies in hepatocytes that were transduced with different FXR isoforms suggested that the FXRα2 isoform promotes fatty acid oxidation and ketone body secretion by stimulating the expression of PPARα and CREB3L3 along with increased expression of PPARα/CREB3L3 target genes involved in fatty acid oxidation and ketogenesis [116]. Currently, to what extent the FXRα2 isoform plays a role in regulating hepatic fatty acid oxidation and ketogenesis at physiological levels of expression remains unclear [117,118].

Effect of fasting on the human liver

Data abound indicating that fasting raises plasma levels of ketone bodies in humans, suggesting that similar mechanisms likely underlie the induction of fatty acid oxidation and ketogenesis in human and murine livers. However, due to obvious ethical constraints, there is a lack of direct insight into the effect of fasting on metabolic pathways and gene expression in the human liver. The use of *in vitro* model systems has not been able to fill that gap, as it is extremely difficult to mimic the *in vivo* fasting response through *in vitro* culture conditions.

A model system of particular interest is the hepatocyte humanized mouse. In these chimeric mice, the liver mainly consists of human hepatocytes. Importantly, a substantial number of mouse hepatocytes are still present, allowing parallel analysis of the effect of fasting on murine and human liver cells [12,62,94]. Analysis of the liver transcriptome shows the concurrent induction of *ACOX1*, *CPT1A*, *HADHA*, *HMGCS2*, *PDK4*, and *PPARGC1A* by fasting in murine and human liver cells [12,94]. Fasting only induced genes involved in peroxisomal fatty acid oxidation in murine liver cells, except for *ACOX1*. Since the aforementioned genes are all PPARα targets, the data suggest that PPARα is activated and mediates gene regulation during fasting in human livers.

Other pathways involved in the regulation of hepatic fatty acid oxidation and ketogenesis

As discussed earlier, studies in hepatocytes obtained from various species showed that glucagon stimulates fatty acid oxidation and ketogenesis [43–46,119]. Studies in glucagon-receptor-deficient mice confirm the stimulatory effect of glucagon via the glucagon receptor on hepatic fatty acid oxidation, which was most evident in the fasted state and is mediated by the activation of PPARα and its target genes [119]. Although glucagon does not affect PPARα mRNA and protein levels [119,120], it causes a redistribution of phosphorylated PPARα to the nucleus leading to enhanced PPARα transcriptional activity, which is mediated by an AMP-activated protein kinase (AMPK)- and p38 mitogen-activated protein kinase (MAPK)-dependent pathway. The stimulatory effect of glucagon on fatty acid oxidation via PPARα was verified in PPARα-deficient hepatocytes [119]. It has also been proposed that the stimulation of fatty acid oxidation by glucagon is mediated by extracellular cAMP, which reportedly promotes PPARα activity through direct phosphorylation by AMPK [121]. In addition, glucagon may stimulate the dephosphorylation and nuclear translocation of histone deacetylase (HDAC)5, stimulating the interaction between HDAC5 and PPARα and promoting PPARα transcriptional activity [122]. Recently, based on the threshold concentration of glucagon required to activate adenylate cyclase, it was questioned whether the stimulation of fatty acid oxidation by glucagon is mediated by cAMP [123]. Instead, it may be mediated by the PLC/IP3/Ca^{2+}/CaM pathway. In apparent conflict with the data described earlier, antibody-mediated or genetic inactivation of the glucagon receptor did not affect fasting plasma ketones [124], thus questioning the role of glucagon in fasting-induced activation of PPARα and ketogenesis in the liver.

Hepatic ketogenesis has also been suggested to be regulated by mammalian target of rapamycin complex (mTORC)1 [125]. mTORC1 is one of the two independent mTOR complexes. It is activated by nutrients and growth factors and accommodates cell growth by promoting the synthesis of macromolecules such as proteins and lipids. Fasting is associated with the suppression of mTORC1 signaling, which is mediated by the tuberous sclerosis complex (TSC), a negative regulator of mTORC1 composed of TSC1 and TSC2. It was reported that TSC1-deficient mice have reduced ketone body production and ketogenic gene expression after fasting, suggesting that mTORC1 suppresses hepatic ketogenesis [125]. However, others have not found decreased ketogenesis upon deletion of TSC1 [126,127], while observing an almost complete inhibition of ketogenesis upon deletion of carnitine O-palmitoyltransferase (CPT)2 [127]. TSC1 deficiency in hepatocytes also did not influence plasma ketones in a mouse model of insulin deficiency, whereas TSC1 deficiency in the entire liver did lead to reduced plasma ketone body levels [128]. Combined deficiency of RAPTOR/AKT2, which leads to decreased mTORC1 signaling, was accompanied by decreased plasma ketones [126]. Overall, the evidence indicating a role of mTORC1 in hepatic ketogenesis is inconsistent.

Sex differences in hepatic fatty acid oxidation and ketogenesis

Sexual dimorphism in plasma ketones

Sex differences in lipid metabolism are well recognized, particularly for hepatic and peripheral lipid handling during various metabolic challenges, including fasting and exercise, as well as in disease states such as obesity [129–131]. During fasting, females exhibit higher plasma ketones than males, as observed in lean individuals [body mass index (BMI) ≤25] [132–139], and individuals with obesity (BMI >25) [133,140,141]. Evidence contradicting these findings exists but is limited [142,143]. Statistically significant differences in plasma ketone body levels have been described as early as after 10 h of fasting [138], yet mostly reach statistical significance beyond 24 h of fasting and persist until the end of multiday-fasting periods, with exceptions [133,134,144]. Integrating circulating β-hydroxybutyrate and NEFA levels and metabolic hormones from fasting studies that collected postprandrial, overnight (10–16 h), 1 day (20–30 h), 2 days (38–48 h), and 3 days (62–72 h) fasting plasma parameters from lean, healthy females and males confirm a faster increase and higher final plasma levels of β-hydroxybutyrate in women compared with men [36–38,131–139,144–157]. Indeed various ^{13}C-palmitate tracer studies documented higher rates of ketogenesis in fasting young lean individuals (BMI ≤25) [136], middle-aged individuals with obesity [141,158], and subjects with familial combined hyperlipidemia [158], indicating that elevated ketone body levels in females are likely due to higher rates of ketone body production. Whether the elevated ketogenesis in females is also driven by differences in the expression of ketogenic and ketolytic genes is unknown, due to obvious ethical constraints.

In line with the human data, studies in mice have reported higher circulating β-hydroxybutyrate levels in female mice compared with male mice after different durations of fasting [159,160], or several weeks of ketogenic diet feeding [161]. Data allowing direct comparison of male and female ketonemia beyond 24 h fasting, or data on sexual dimorphism in expression levels of relevant genes are not available.

Sexual dimorphism in plasma NEFAs

The higher plasma β-hydroxybutyrate levels in females versus males are accompanied by higher NEFA levels in healthy lean women compared with men [136,138], as well as in women with diabetes [162], and obesity [140], and have been reported as early as after 10–12 h of fasting and up to 72 h in lean women and 144 h in women with obesity [133,134]. Integrating fasting NEFA levels across studies [36–38,131–139,144–157] confirms higher NEFA levels in women,

although we only found one study that reported NEFA levels at subsequent time points. Measurements of glycerol appearance in the plasma suggest that women have higher fasting plasma NEFA levels due to higher whole-body lipolytic rates [139,144,163]. The higher rate of lipolysis in women is likely driven by a combination of anatomical differences in total and regional fat mass as well as sensitivity towards lipolysis-modulating hormones, as recently reviewed elsewhere [164]. Although fasting plasma NEFAs thus appear to be higher in women than in men, this conclusion cannot be drawn for female versus male mice. The limited number of published studies reporting fasting plasma NEFA levels in male and female mice do not convey a clear and consistent picture [165–169].

Della Torre *et al*. found higher malonyl-CoA levels in female mouse livers after a 6-h fast [170]. As malonyl-CoA inhibits CPT1, this might lead to lower rates of fatty acid oxidation in the female liver. How the higher malonyl-CoA levels connect to enhanced ketonemia in females is unclear. The observations that female mouse livers accumulate more triglycerides than male mouse livers [170], while the opposite has been shown in humans [142], suggest that fasting livers in female mice and humans compartmentalize incoming fatty acids differently.

Role of hormonal factors in sexual dimorphism in plasma ketones and NEFA

Besides differences in plasma NEFA levels, it could be hypothesized that the higher fasting plasma ketones in females versus males are due to direct hormonal influences. Integrating measurements from various fasting studies [36–38,131–139,144–157], suggests similar relative changes in metabolic hormones levels in the two sexes. This result is in line with studies that compared these hormones head-on in a single study and documented no statistically significant differences [133,134,139,145]. It has been reported that plasma glucagon more strongly increases during fasting in women than in men, at least in the initial phase of the fast [137,140], but these reports are inconsistent [134], and not supported by our analysis. The sexual dimorphism in plasma ketone body and NEFA levels is unlikely due to differences in the levels of metabolic hormones between the two sexes.

Extensive periods of food deprivation have been linked to compromised reproductive function. In young lean females, fasting in the follicular phase may lead to extended follicular phases and anovulation [171], as well as increased levels of estradiol during the follicular phase [146,171], which in turn may influence the metabolic response to fasting [172]. Unfortunately, we only found three studies that investigated differences in fasting-induced circulating NEFA and/or ketone body levels in the same individual in subsequent phases of the menstrual cycle [132,173,174]. Negative findings of the follicular phase (high estrogen) versus luteal phase (low estrogen) on NEFA levels, NEFA flux, and ketone body levels after an overnight fast are contrasted by the positive findings of Corssmit *et al.* after 1 day of fasting [132,173,174]. While the latter study did not measure ketone bodies, it documented a higher increase in circulating NEFAs, a higher rate of fatty acid oxidation, and a lower respiratory quotient in women fasting in the follicular phase (day 5) compared with the luteal phase (day 24) [132]. Long-term users of oral combination contraceptives showed even stronger responses in the abovementioned parameters to a 24-h fast [132]. An elevated fasting response in plasma NEFAs or ketone bodies has also been reported in other studies that compared oral combination contraceptive users with women fasting in the luteal phase [174,175], or the follicular phase [138,146], after 10–86 h fasting. In summary, these findings suggest that elevated estradiol levels, either endogenously through menstrual phases or via exogenous contraceptives, augment fasting NEFA and ketone body levels, which is in line with rodent data showing that estrogen administration stimulates fasting-induced ketonemia [176,177].

In men, fasting has been shown to reduce circulating luteinizing hormone, follicle-stimulating hormone, and testosterone levels [178,179]. In addition to its important function in the development of male sex characteristics, testosterone exerts a host of metabolic effects in healthy men, including dose-dependent increases in anabolic balance, lean muscle mass, muscular strength, and fat oxidation [180–182]. *In vitro* studies indicate that testosterone impairs catecholamine-induced lipolysis in human subcutaneous adipocytes by suppressing hormone-sensitive lipase (HSL) expression and activation [183]. Accordingly, the reduction in plasma testosterone levels during fasting might contribute to the increase in circulating NEFA levels in men. Overall, convincing evidence that fasting-induced changes in testosterone levels modulate NEFA and ketone body levels is lacking, as is evidence pointing to a direct effect of testosterone on hepatic fatty acid oxidation and ketogenesis. It can, however, be hypothesized that the inhibitory effect of testosterone and stimulatory effect of estrogen on adipose tissue lipolysis may contribute to the sexually dimorphic response in plasma NEFA and ketone body levels in humans during fasting.

Sexual dimorphism in transcriptional regulation of hepatic fatty acid oxidation and ketogenesis

Studies have shown sexual dimorphism in PPARα-dependent regulation, including under normal breeding conditions [184], in response to perfluorooctanoic acid (PFOA) [185], in response to pharmacological inhibition of mitochondrial fatty acid import [186], as well as in various models of diet-induced nonalcoholic fatty liver disease (NAFLD) [160]. For instance, it was observed that the effect of PPARα deficiency on plasma triglycerides was more pronounced in female mice, whereas the effect of PPARα deficiency on liver triglycerides was more pronounced in male mice [184]. Hence, it could be hypothesized that the generally higher plasma ketone body levels in female versus male humans and mice may be due to a sexually dimorphic PPARα-dependent regulation of hepatic fatty acid oxidation and/or ketogenesis. Supporting this notion, the expression of *Ppara*, *Cpt1a*, and *Fgf21* was found to be higher in 24-h-fasted female than male mice [168]. By contrast, Jalouli and colleagues found higher levels of hepatic *Ppara* mRNA and protein in male compared with female rats in the fed and fasted state. Fasting increased hepatic *Ppara* mRNA levels to a similar degree in both sexes. Gonadectomy decreased *Ppara* mRNA expression to a similar level extent in both sexes, whereas hypophysectomy increased *Ppara* mRNA to a larger extent in females than in males [187].

Using hepatocyte-specific PPARα-deficient mice, Smati and colleagues investigated whether the PPARα-driven changes in hepatic lipid metabolism and gene expression during fasting are sexually dimorphic [160]. Compared with their wild-type littermates, 16-h-fasted, hepatocyte-specific, PPARα-deficient male mice, but not female mice, showed increased hepatic triglycerides. Transcriptomics analysis revealed that the number of genes affected by PPARα deficiency was much higher in females than in males. Many of the genes specifically altered by PPARα deficiency in female mice were linked to inflammation-related pathways [160]. No evidence was provided indicating a more pronounced impact of PPARα deficiency on genes involved in fatty acid oxidation and ketogenesis in female than in male mice.

Altogether, women and female mice present with elevated ketonemia during fasting compared with their male counterparts, yet there is insufficient evidence for sexual dimorphism in the transcriptional regulation of fatty acid oxidation and ketogenesis by PPARα. No studies were found that investigated sexual dimorphism in the regulation of hepatic lipid metabolism by CREB3L3 or any of the other factors discussed earlier.

Concluding remarks

In recent years, fasting regimens have gained popularity for their potential beneficial effects on health and weight control. To be able to effectively utilize fasting regimens for therapeutic

Outstanding questions

To what extent and how does fasting influence transcriptomic, proteomic, and metabolomic responses in non-hepatocytes in the liver? How important is the crosstalk between all resident cell populations for the overall hepatic fasting response?

Regulatory transcriptional mechanisms are defined by complex systems of transcription factor interplay. What can we further learn about the complex transcriptional mechanisms through cell-targeted transcription factor knockout studies?

What can novel technologies in human model systems teach us about the potential similarities and differences between mouse and human liver metabolism during fasting?

purposes, a thorough understanding of the molecular processes governing the metabolic response to fasting is required. In this review, we presented the latest insights into the mechanisms underlying the induction of fatty acid oxidation and ketogenesis in the liver by fasting. Despite the major advancements in our understanding of the signaling pathways triggered by fasting, several knowledge gaps remain (see Outstanding questions).

First, while we understand reasonably well what happens in hepatocytes during fasting, there is minimal insight into the impact of fasting on other cell types in the liver, including Kupffer cells, endothelial cells, stellate cells, and cholangiocytes. Also, it is unclear to what extent the metabolic response to fasting differs among hepatocytes, and may depend on factors such as zonation and contact with adjacent non-hepatocytes. Single-cell and/or spatial omics approaches will enable further understanding of the cellular and spatial organization of hepatic metabolic processes during fasting.

Second, it is well established that PPARα is the master regulator of lipid metabolism in the liver during fasting. Other transcription factors such as CREB3L3, FOXA2, GR, as well as glucagon-induced signaling, may cooperate with or influence PPARα activity and/or expression, but their individual impact appears to be relatively limited, although further study is needed. In particular, comparative liver transcriptomics analysis of the impact of single or combined hepatocyte-specific deletion of the abovementioned pathways should help clarify the relative importance and interdependence of these pathways in the transcriptional regulation of hepatic fatty acid oxidation and ketogenesis.

Third, studies in mice and other human models have established the key molecular pathways governing hepatic fatty acid oxidation and ketogenesis. Further advancement in the use of human model systems, including hepatocyte-humanized mice and human liver organoids, in combination with the application of cell-specific gene targeting methods, should further clarify the molecular mechanisms regulating fatty acid oxidation and ketogenesis in the human liver.

Fourth, women and female mice display higher levels of fasting ketonemia than their male counterparts. While these differences may be explained by higher NEFA availability in females during fasting, other explanations have not been sufficiently explored. In particular, the impact of endogenous male sex hormones on hepatic lipid metabolism during fasting is unclear.

Major insights have been gained into the intricate regulation of hepatic fatty acid metabolism during fasting. It is expected that new advances in technology will allow filling in several remaining pertinent questions.

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Declaration of interests

No interests are declared.

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