Chapter

# Metabolic Responses to Energy-Depleted Conditions

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## Abstract

Dietary intervention is one of the most important approaches for the treatment of metabolic diseases such as diabetes mellitus. Fasting and caloric restriction have profound effects on systemic metabolism. The energy source-producing organs, such as the liver, and peripheral tissues rewire their metabolism to meet the energy demands of the whole body. Glycogenolysis, fatty acid oxidation, and ketone body production are characteristic metabolic changes that occur during fasting and caloric restriction. These metabolic changes are regulated by various signaling cascades including PPARα and FGF21. Moderate fasting and caloric restriction have also been implicated in extending the lifespan in a variety of organisms from nematodes to vertebrates. Intensive research has unveiled several regulatory mechanisms of longevity including metabolic regulators such as mTOR and sirtuins. The epigenome has been attracting attention as a mechanism underlying metabolic diseases and longevity. The epigenome is the concept that involves covalent modifications of DNA, histones, and RNA, which are mediated by the action of epigenetic enzymes. The activity of these enzymes is regulated by energy states, i.e. metabolites including ketone bodies and intermediates of various metabolic pathways. Thus, energy states are recorded in cells as an epigenetic memory, which may cause future onset of metabolic diseases and affect lifespan.

**Keywords:** Fasting, caloric restriction, diabetes mellitus, obesity, glycolysis, the TCA cycle, fatty acid oxidation, ketone body, PPARα, FGF21, insulin, glucagon, longevity, metabolites, and epigenome

## 1. Introduction

For the treatment of diabetes mellitus, dietary intervention is one of the most important basic approaches along with exercise therapy. Since dietary guidance for diabetes is often based on limiting caloric intake, it is important to understand the effects of fasting and caloric restriction on systemic metabolism. Furthermore, the effects of the Mediterranean diet and the pros and cons of carbohydrate-restricted diets have recently attracted attention, and it is now widely recognized that the proportion of certain nutrients in the diet and the order in which they are eaten can affect nutrient absorption and systemic metabolism. In countries where excessive food supply has caused obesity and the associated diseases, many people have adopted fasting and caloric restriction for weight control. Academic studies have shown that moderate caloric restriction has a positive effect on those diseases and

contributes to longevity through anti-aging effects and prevention of age-related diseases. However, given that excessive fasting or caloric restriction can lead to malnutrition, it is important to accurately understand the effects on systemic metabolism.

During fasting, carbohydrates, fats, and proteins are utilized as energy sources in many organs. However, since the brain cannot directly utilize fatty acids derived from lipolysis, the insight into energy metabolism in the brain is essential to understand the systemic metabolism during fasting. In the early stages of fasting, glucose is provided to the brain by glycogenolysis (**Figure 1A**). In the case of prolonged fasting, gluconeogenesis is activated in the liver to produce glucose from pyruvate and lactate, as well as from glycerol produced by lipolysis and from amino acids produced by proteolysis (**Figure 1B**) [1]. When fasting is further prolonged, glucose is supplied by renal gluconeogenesis, and ketone bodies produced by fatty acid oxidation (or β-oxidation), and acetate are used as energy sources for the brain and skeletal muscles (**Figure 1A**) [2, 3]. Thus, brain activity is maintained by glucose and ketone bodies from the multiple sources. Of note, most of gluconeogenesis takes place in the liver and to a lesser extent in the proximal tubules of the kidney, and ketone bodies are mainly produced in the liver.

It has long been known that fasting and caloric restriction are associated with an extended lifespan in many organisms. Longevity is regulated by several factors such as mechanistic target of rapamycin (mTOR), sirtuins (SIRTs), AMP-activated protein kinase (AMPK), forkhead box protein O (FOXO), and growth hormone (GH)/insulin-like growth factor-1 (IGF-1), but the mechanism by which these factors extend lifespan in humans is not yet fully understood. One possible hypothesis is that fasting and caloric restriction are memorized in the epigenome of cells. More specifically, metabolites produced during fasting serve as inhibitors or substrates of epigenetic enzymes. For example, ketone bodies mainly inhibit class I histone deacetylases (HDACs) to promote histone acetylation. In addition, acetyl-CoA, produced by fatty acid oxidation, and α-ketoglutarate (α-KG, also known as 2-oxoglutarate), an intermediate metabolite of the tricarboxylic acid (TCA) cycle (also known as the citric acid cycle or the Krebs cycle), are substrates for histone acetyltransferases and epigenetic demethylases, respectively. Thus, the states of energy metabolism, including fasting, are recorded in cells as epigenomic memories, which may cause the development of future diseases.

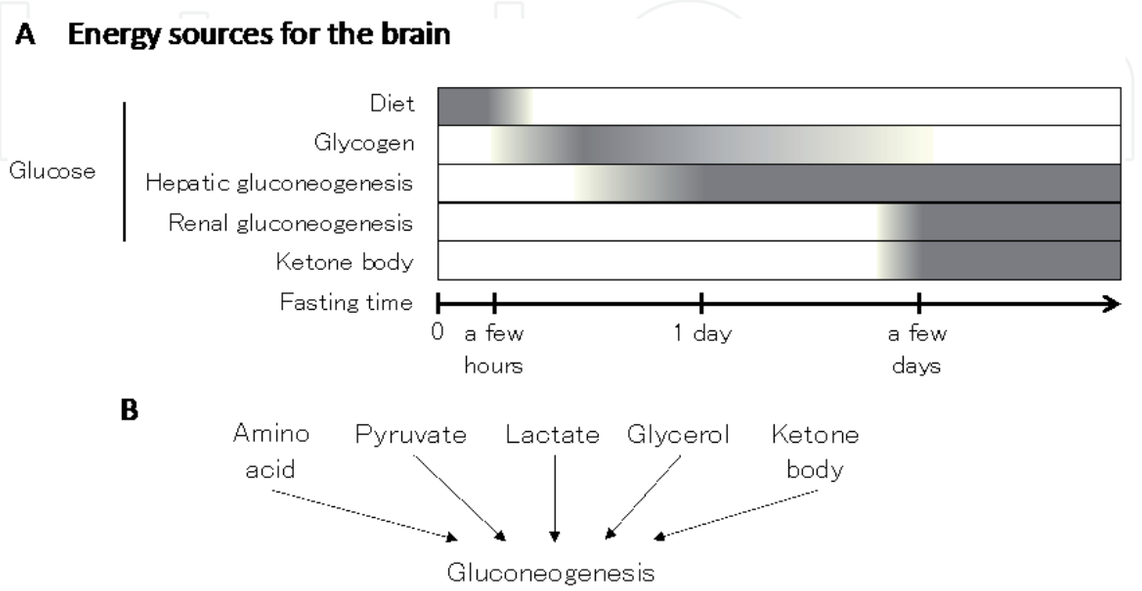



**Figure 1.**
*The brain energy sources under the fasting condition. A. Energy sources for the brain, B. Energy sources for gluconeogenesis.*

## 2. Energy storage in the body

Under normal metabolic conditions, some of the excess glucose is stored as glycogen, mainly in the liver and muscles, and further excess energy is stored as fat mainly in the adipose tissue. Fat in the body consists of triglycerides (TGs), which are transported to the adipose tissue in the form of chylomicrons from the intestine, and also produced from excess glucose in the adipose tissue and the liver. Under the feeding conditions, glucose is metabolized mainly in adipocytes and hepatocytes via glycolysis to produce pyruvate, which is then converted to acetyl-CoA and combined with oxaloacetate to enter the TCA cycle as citrate in mitochondria. Excess citrate is transported to the cytoplasm and converted to acetyl-CoA, which is then used as a substrate for fatty acids synthesis via malonyl CoA produced by the rate-limiting enzyme, acetyl-CoA carboxylase. Fatty acids are esterified with glycerol to produce triglycerides and stored in the adipose tissue.

## 3. Energy supply under fasting conditions

The liver, muscles, adipose tissues, and brain are all closely involved in energy metabolism and are important organs for understanding whole body metabolism in the fasting state. The brain cannot directly utilize fat because it is not capable of fatty acid oxidation unlike many other organs. Therefore, during prolonged fasting, glucose is supplied to the brain from multiple sources to maintain its functions. In addition, ketone bodies are produced as an energy source for the brain during extremely long fasting. The reason why the brain is unable to oxidize fatty acids remains controversial, but it is thought to be because fatty acids cannot cross the blood–brain barrier and the brain does not have the enzymes necessary for β-oxidation.

In the normal state of energy metabolism under feeding conditions, dietary sugar is commonly used as an energy source in all organs. Glucose is oxidized via glycolysis to eventually yield two molecules of pyruvate. In the presence of oxygen, pyruvate enters the TCA cycle, and it is completely oxidized to produce six molecules of carbon dioxide. At this step, $NAD^+$ and FAD are reduced to produce NADH and $FADH_2$, respectively, and are transported to the respiratory chain of mitochondria to produce energy in the form of ATP.

In the early stage of fasting, stored glycogen, a branched polymer of glucose, is degraded to provide glucose via glucose 6-phosphate (**Figure 2**). Glycogen-derived glucose is used for energy supply by the glycolytic pathway and the TCA cycle in most of organs except the liver, muscles, and adipose tissue. However, the amount of energy stored as glycogen is consumed in a day of fasting, and hepatic glycogen is almost completely depleted after two to three days of starvation. The degradation of glycogen proceeds as phosphorylase sequentially removes glucose monomers by cleaving the α-(1 → 4) bond in a phosphate-dependent manner. At the branched end of glycogen, when degradation proceeds to four glucose residues near the α-(1 → 6) bond, three glucose molecules at the branching end are transferred to the other chain end by a group of glycogen debranching enzymes. The remaining glucose molecules are then hydrolyzed to remove them, and further degradation by phosphorylases continues. The glucose 1-phosphate produced by phosphorylase is converted to glucose 6-phosphate by phosphoglucomutase and enters the glycolytic pathway (**Figure 2**). Glucose 6-phosphate is converted to glucose by the action of glucose 6-phosphatase (G6Pase) in the liver, and then released into the bloodstream via Glut2 to supply glucose to the brain and other organs. On the other hand, G6Pase is not expressed in the muscles, so stored glycogen is used only for local energy production.

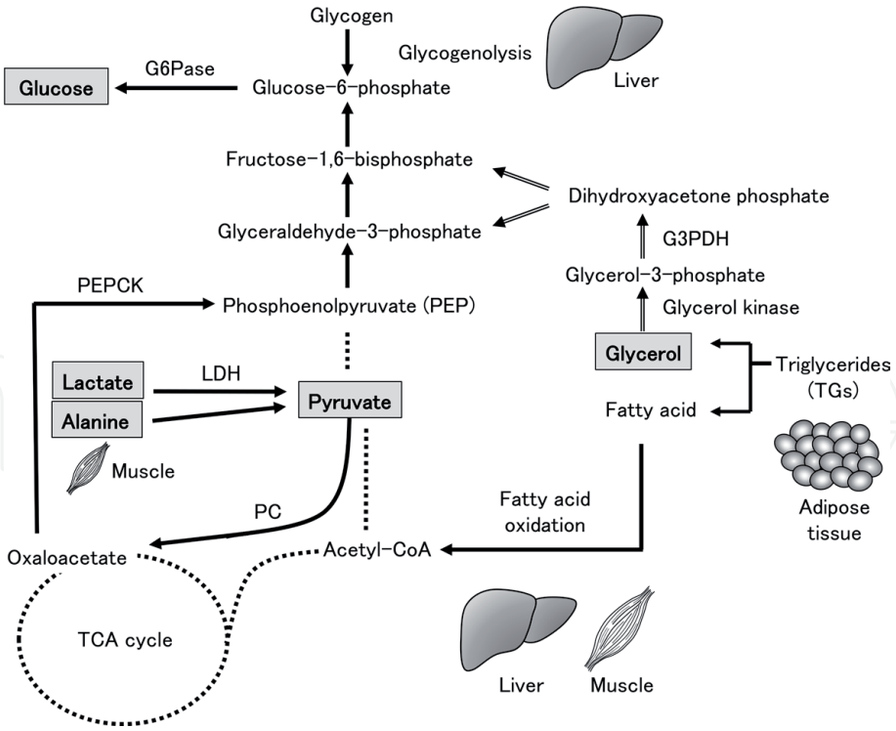



**Figure 2.**
*Gluconeogenesis under the fasting condition.*

During fasting, TGs in the adipose tissue are also degraded and used for fatty acid oxidation (**Figures 2** and **3**). TGs are broken down into fatty acids and glycerol by hormone-sensitive lipase (HSL) and a rate-limiting enzyme, adipose triglyceride lipase (ATGL). Fatty acids are bound to albumin and transported to the liver and muscles, where they are taken up by facilitated transport for fatty acid oxidation. Long-chain fatty acids taken up by the cells pass through the mitochondrial membrane to the matrix. Fatty acids are converted to acyl-CoA by acyl-CoA synthase and then conjugated with carnitine to form acyl-carnitine by carnitine palmitoyltransferase (CPT1) on mitochondrial outer membrane (CPT1a: mainly in the liver, CPT1b: mainly in the skeletal muscle and brown fat) and pass through mitochondrial inner membrane to the matrix via carnitine-acylcarnitine translocase (CACT). Fatty acids are then released from carnitine through the action of CPT2 on mitochondrial inner membrane (**Figure 3**). In mitochondrial matrix, fatty acids are continuously oxidized via FAD-dependent acyl-CoA dehydrogenase to produce $FADH_2$, $NADH+H^+$, and acetyl-CoA (**Figure 3**). Although fatty acid-derived acetyl-CoA enters the TCA cycle for energy production, it does not contribute to gluconeogenesis because two carbon atoms derived from the acetyl-CoA are removed in the TCA cycle. In contrast, the glycerol produced by lipolysis can be used as a substrate for gluconeogenesis (**Figure 2**).

When fasting persists, glucose is supplied to multiple organs via gluconeogenesis in the liver. Gluconeogenesis is particularly important to provide glucose as an energy source for the brain, which is unable to oxidize fatty acids. In the liver, gluconeogenesis is carried out from amino acids, pyruvate, lactate, glycerol, and ketone bodies (**Figures 1B** and **2**). During several weeks of starvation, about 80 grams of glucose is produced daily via gluconeogenesis, of which 15–20 grams is derived from amino acids, mainly alanine, 35–40 grams from pyruvate and lactate, 20 grams from glycerol from fat, and 10 grams from ketone bodies [1]. Amino acids are supplied by degradation of proteins in the muscles during fasting. Alanine is transported to the liver via the bloodstream, and is then converted to pyruvate for gluconeogenesis (**Figure 2**). Pyruvate is metabolized to oxaloacetate by pyruvate carboxylase (PC)

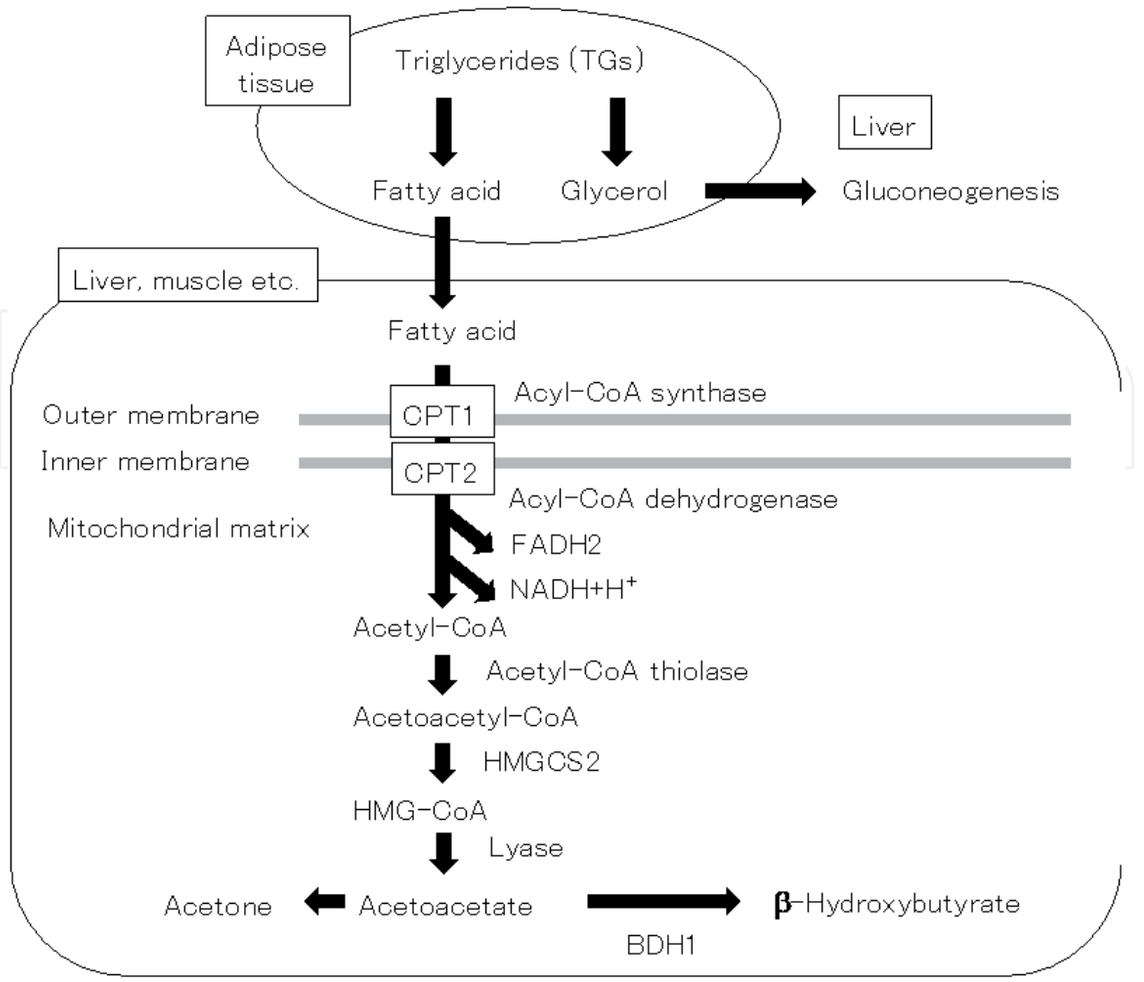



**Figure 3.**
*Lipid metabolism and ketone body production during fasting.*

and then decarboxylated by phosphoenolpyruvate carboxykinase (PEPCK) to produce phosphoenolpyruvate (PEP) (**Figure 2**). PEP is an intermediate product of the glycolytic pathway, and the following gluconeogenic process utilizes the glycolytic enzymes except for the step of fructose-1,6-bisphosphate to fructose-6-phosphate which requires fructose-1,6-bisphosphatase [4] and the step of glucose-6-phosphate to glucose mediated by G6Pase. Lactic acid is converted to pyruvate by lactate dehydrogenase (LDH) for gluconeogenesis (**Figure 2**). Glycerol is phosphorylated by glycerol kinase in the liver to produce glycerol 3-phosphate, which is then oxidized by glycerol 3-phosphate dehydrogenase (G3PDH) to dihydroxyacetone phosphate, an intermediate of the glycolytic pathway. Dihydroxyacetone phosphate undergoes gluconeogenesis via glyceraldehyde 3-phosphate or fructose 1,6-diphosphate (**Figure 2**). Furthermore, under long-term starvation for several days or a week where water, vitamins, salt, and other minerals are supplied, glucose production occurs not only in the liver but also in the renal cortex, which is responsible for about 40% of total body glucose production [1].

Gluconeogenesis is tightly regulated by the products of fatty acid oxidation such as $NADH+H^+$, acetyl-CoA, and ATP (**Figure 4**). In normal glucose metabolism, pyruvate enters the TCA cycle via acetyl-CoA by the action of pyruvate dehydrogenase (PDH). However, during fasting, pyruvate is converted to oxaloacetate by PC for gluconeogenesis as described above (**Figure 4**). The activity of PC is stimulated by acetyl-CoA. In addition, $NADH+H^+$, acetyl-CoA, and ATP inhibit PDH activity (**Figure 4**). Furthermore, $NADH+H^+$ inhibits isocitrate dehydrogenase (IDH), one of the enzymes responsible for the TCA cycle (**Figure 4**). Thus, $NADH+H^+$,

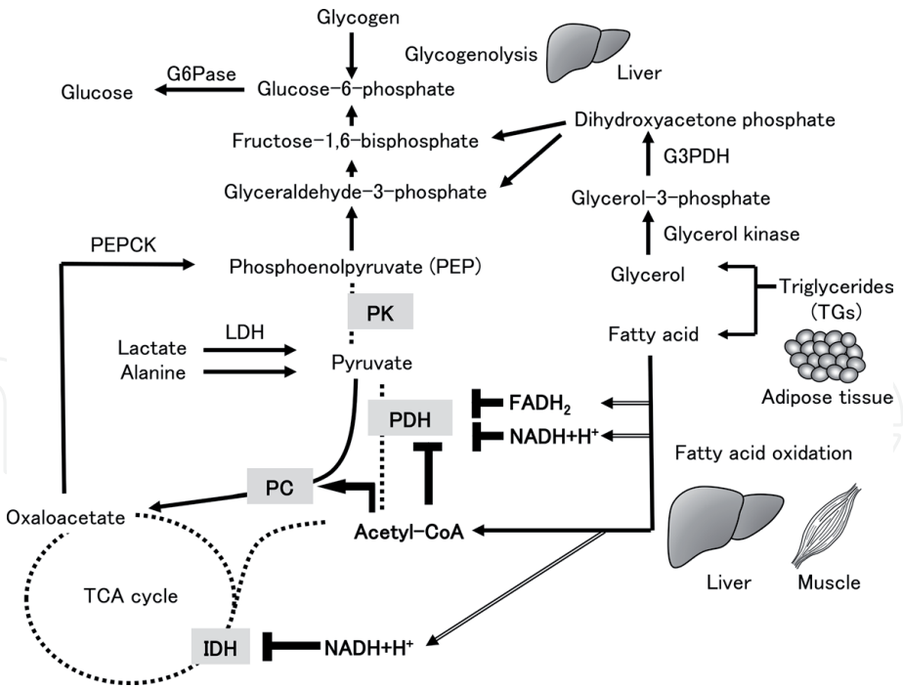



**Figure 4.**
*Fatty acid oxidation and gluconeogenesis during fasting.*

acetyl-CoA, and ATP, which are generated by fatty acid oxidation, regulate the activities of enzymes in the gluconeogenesis pathway, and these regulatory mechanisms ensure a consistent flow of metabolites for energy supply through gluconeogenesis and fatty acid oxidation during fasting. Since PEP produced by PEPCK is an intermediate product of the glycolytic pathway, it could theoretically be converted to pyruvate and re-enter the TCA cycle. However, because pyruvate kinase (PK) in the liver is inhibited by alanine and inactivated by protein kinase A (PKA), which is activated by glucagon, PEP produced during fasting is used for gluconeogenesis but not glycolysis. In other words, gluconeogenesis and amino acid metabolism are consistently regulated under fasting conditions. In addition, nitrogen sources stored in the muscles are used for gluconeogenesis in the renal cortex under prolonged fasting. Glutamine and alanine are metabolized from branched-chain amino acids such as leucine, isoleucine, and valine in the muscles, and released into the bloodstream. Glutamine is then primarily used for gluconeogenesis via the TCA cycle in the renal cortex. This indicates that diet therapies that focus primarily on fasting result not only in burning of stored fat but also loss of muscle mass.

## 4. Ketone body as an energy source during long-term fasting

Under glucose-depleted conditions during fasting, the brain and muscles use ketone bodies and acetate as energy sources other than glucose (**Figure 1B**) [3, 5]. These energy sources are critical to sustain function of the brain because it cannot directly metabolize fatty acids. A human study investigated the energy sources of the brain under long-term starvation for 5–6 weeks. When only water, vitamins, and minerals such as salt are supplied, urinary nitrogen excretion, an indicator of amino acid-derived gluconeogenesis, dropped to about 4–5 grams per day, and two-thirds of the energy source of the brain comes from β-hydroxybutyrate and acetoacetate [1].

The metabolic systems that use ketone bodies as an energy source can be traced back to bacteria, archaea, and protozoa. In humans, most ketone bodies are produced in the liver. During fasting, the most part of acetyl-CoA produced by fatty

acid oxidation is used for ketone body production, while only the limited amount enters the TCA cycle (**Figure 3**). At the first step of the TCA cycle, acetyl-CoA is conjugated to oxaloacetate through the action of citrate synthase to produce citrate. However, oxaloacetate is relatively scarce during fasting because it is consumed by glucose production. As a result, excess acetyl-CoA produced by fatty acid oxidation is used exclusively for ketone body production. In addition, $NADH+H^+$, acetyl-CoA, and ATP, which are produced by fatty acid oxidation, regulate gluconeogenesis-related enzymes to stimulate gluconeogenesis. In addition, $NADH+H^+$ suppresses the activity of IDH in the TCA cycle, which in turn suppresses the TCA cycle and directs fatty acid-derived acetyl-CoA toward ketone body production.

Ketone bodies are produced in mitochondria in the following reactions: two molecules of acetyl-CoA are combined by acetoacetyl-CoA thiolase to produce acetoacetyl-CoA, and an additional molecule of acetyl-CoA is conjugated by HMG-CoA synthase 2 (HMGCS2) to produce HMG-CoA, which is then cleaved by lyase to form acetoacetate (**Figures 3** and **5**). Acetoacetate can be converted to acetone through spontaneous non-enzymatic decarboxylation or to β-hydroxybutyrate (D-3-hydroxybutyrate) by 3-hydroxybutyrate dehydrogenase (BDH1) (**Figure 3**). β-Hydroxybutyrate is the most abundant ketone body in the blood. Here, the irreversible reaction by HMGCS2 is a key reaction for ketone body production, and the activity of BDH1 is increased by $NADH+H^+$.

The basal level of β-hydroxybutyrate in humans is at the level of a few μM under feeding conditions, and the blood concentration increases to 200–300 μM after 12–16 hours of fasting, 1–2 mM after 2 days of fasting, and as high as 6–8 mM after prolonged fasting [6]. Ketone bodies also reach more than 2 mM with a ketogenic diet that excludes most carbohydrates, and intense exercise for about 90 minutes also increases ketone bodies to 1–2 mM. In neonates, the production and utilization of ketone bodies are more efficient than adults. The serum concentration of ketone bodies is as high as 2–3 mM just after birth, and the neonatal brain uses ketone bodies as an important energy source.

Ketone bodies are produced by the liver and supplied to the brain, muscles, and kidneys during fasting, but the liver cannot utilize ketone bodies as an energy source because it does not express 3-keto acid CoA transferase (OXCT1/SCOT). β-Hydroxybutyrate is produced in the liver and released into the bloodstream via

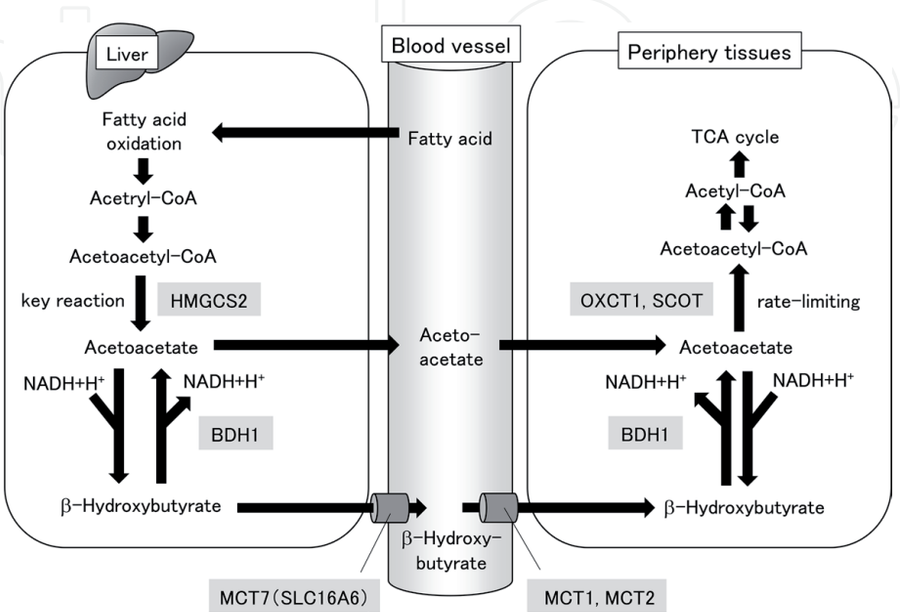



**Figure 5.**
*Production and utilization of ketone bodies.*

monocarboxylic acid transporter 7 (MCT7/SLC16A6) (**Figure 5**) [6]. During prolonged fasting, the high concentration of β-hydroxybutyrate in the blood is taken up by the brain through the blood–brain barrier via several monocarboxylic acid transporters, including MCT1 and MCT2 (**Figure 5**). Once taken up into neurons, β-hydroxybutyrate reverses the ketone body production pathway to produce acetoacetate by BDH1 and further converts to acetoacetyl-CoA by OXCT1/SCOT. The activity of BDH1 is promoted by $NADH+H^+$ produced during fatty acid oxidation (**Figures 3** and **5**). In addition, the reaction of OXCT1/SCOT is the rate-limiting step to produce acetoacetyl-CoA, which is accompanied by the production of an intermediate metabolite of the TCA cycle, succinate, in a succinyl CoA-dependent manner (**Figure 5**).

In addition, acetate is also utilized as an energy source during prolonged fasting. In the liver, acetate is produced from acetyl-CoA by the action of acetyl-CoA hydroxylase during fasting, and is released from the liver to other organs. Acetate is utilized as an energy source for acetyl-CoA production via type 2 acetyl-CoA synthase (AceCS2), which is particularly abundant in mitochondria of the muscles [2].

## 5. Regulatory mechanisms of ketone body production

HMGCS2 is a key enzyme for the regulation of ketone body production. The expression of HMGCS2 is strongly regulated by forkhead box protein A2 (FOXA2), peroxisome proliferators-activated receptor α (PPARα), fibroblast growth factor 21 (FGF21), and mTOR, and its activity is enhanced through deacetylation by sirtuin 3 (SIRT3) [6]. FOXA2 binds directly to the promoter region of HMGCS2 and activates its transcription. The expression of FOXA2 is regulated by insulin and glucagon. Insulin-mediated phosphorylation inactivates FOXA2 by translocating it out of the nucleus, while glucagon activates FOXA2 through p300-mediated acetylation, thereby contributing to ketone body production. FOXA2 deacetylation is also regulated by class I and class II HDACs and SIRT1, a class III HDAC. In addition, mTORC1 complex is known to suppress PPARα, and rapamycin promotes ketone body production by inhibiting mTORC1 complex.

## 6. Regulation of metabolism under fasting conditions by PPARα and FGF21

PPARα is a nuclear receptor expressed in the liver, kidney, heart, and brown adipose tissue, which is activated by long-chain fatty acids and involved in fatty acid metabolism (**Figure 6**). PPARα forms a heterodimer with retinol X receptor (RXR) and regulates transcription by binding to the response sequence called PPAR response element (PPRE) in the gene regulatory regions. In the liver, PPARα promotes the expressions of a variety of genes related to β-oxidation, including FGF21, HMGCS2, CPT1a, and acyl-CoA oxidase (ACOX), which is the rate-limiting enzyme for peroxisomal β-oxidation (**Figure 6**).

FGF21, a target gene of PPARα, is a member of the FGF family, with 22 members in humans, and belongs to the same subfamily as FGF15/19 and FGF23 [7, 8]. FGFs in this subfamily characteristically function as hormones. FGF21 is expressed in the liver, adipose tissue, skeletal muscle, and pancreas, and the liver mainly secretes FGF21 as a hormone [9]. The expression of FGF21 is enhanced by fasting and ketogenic (or high-fat) diets [7, 10]. FGF21 levels in infants are higher than fasting FGF21 levels in adults, which is thought to be induced by milk-derived free fatty acids. In addition to PPARα, glucocorticoid receptors, activating transcription factor 4 (ATF4), cAMP response element binding protein

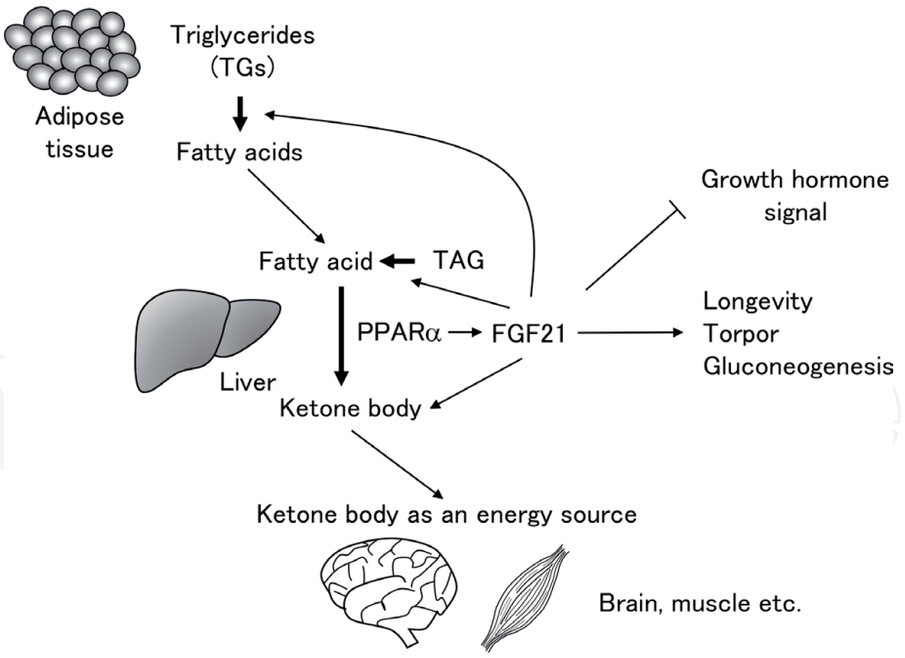



**Figure 6.**
*Regulation of energy metabolism by FGF21.*

H (CREBH), carbohydrate response element binding protein (ChREBP), PPARγ, farnesoid X receptor (FXR), and activin B induce the expression of FGF21 in the liver [11], while liver X receptor (LXR) inhibits the expression [12]. In the skeletal muscles, FGF21 is expressed through ATF4 under specific conditions such as metabolic stresses in mitochondria, and is also regulated by the phosphatidylinositol-3 kinase (PI3K) and Akt signals [12].

FGF21 plays an important role in the regulation of systemic energy metabolism during fasting. In the white adipose tissue, FGF21 induces lipolysis by enhancing the transcription of HSL and ATGL [7]. Free fatty acids produced by lipolysis promote fatty acid oxidation and the protein expression of HMGCS2, which induces the production of ketone bodies (**Figure 6**). FGF21 also enhances the expression of lipolytic enzymes in the liver, suppresses glycogenolysis, and promotes gluconeogenesis, but does not significantly affect glycolysis. FGF21 binds to the FGF receptor (FGFR) and its co-factor β-Klotho on the plasma membrane of target cells, and regulates transcription and translation of the target genes via phosphorylation cascades. FGF21 also regulates glucose metabolism in the liver partially via peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), which acts as a co-activator of transcriptional regulators and induces gene expression related to gluconeogenesis, fatty acid oxidation, and ketone body production during fasting [13]. In rodent models, pharmacological concentrations of FGF21 promote glucose uptake in adipocytes, increase insulin sensitivity, reduce blood triglyceride and hepatic fat, and suppress weight gain on a high-fat diet. In addition, it induces the expression of Glut1 in adipocytes [14], increases the number of pancreatic islets and the content of insulin per islet [15], and reduces blood glucagon levels by suppressing glucagon secretion [14]. In diabetic monkeys, FGF21 lowers blood glucose, serum insulin, triglyceride, and low density lipoprotein (LDL) cholesterol levels, increases high density lipoprotein (HDL) cholesterol levels in the blood, and induces weight loss without inducing hypoglycemia [16]. Human blood FGF21 levels were shown to be elevated in individuals with obesity, type 2 diabetes, and insulin resistance. It is also reported that blood FGF21 levels in hyperlipidemic patients are twice as high as in the normal group, and that administration of fibrate, a PPARα ligand, increases blood FGF21 levels.

In addition, FGF21 levels in humans are increased by prolonged fasting for 7 days [17]. Thus, even under feeding conditions, FGF21 induces a fasting-like metabolic states, such as gluconeogenesis, fatty acid oxidation, and ketone body production (**Figure 6**) [12].

Living organisms suppress GH and reproductive signals to reduce unnecessary energy consumption during fasting. Mice with excess FGF21 show reduced response to GH and suppressed reproductive signals (**Figure 6**) [18, 19]. In detail, FGF21 transgenic mice show reduced phosphorylation of signal transducer and activator of transcription 5 (STAT5) downstream of Janus kinase (JAK) 2 in response to GH in the liver and reduced blood IGF-1 levels [18]. In addition, FGF21 transgenic female mice have a suppressed luteinizing hormone (LH) surge due to inhibition of vasopressin signals in the hypothalamus [19]. The latest statistical analysis, which excluded the contribution of insulin resistance and body fat percentage, showed elevated blood FGF21 levels in human anorexia [20], suggesting that FGF21 may be involved in impaired GH signaling in anorexic patients. In addition, FGF21 plays a role in energy conservation by inducing a hibernation-like state (torpor) in mice [7]. During mouse torpor and squirrel hibernation, in addition to hypothermia and hypoactivity, it is known that pancreatic lipase is ectopically induced outside the pancreas and FGF21 induces the ectopic expression in the liver [7, 21]. The pancreatic lipase is capable of hydrolyzing TGs into glycerol and fatty acids over a wide temperature range, and thus may provide fatty acids as an energy source during torpor and hibernation.

Furthermore, FGF21 transgenic mice exhibit a long lifespan (**Figure 6**) [22]. It is reported that the median lifespan of wild-type mice was 28 months, while that of FGF21 transgenic mice was 38 months. Interestingly, the longevity of the FGF21 transgenic mice did not require restriction of food intake, and insulin sensitivity was maintained even when food intake was increased. This suggests that FGF21 increases lifespan by shifting systemic metabolism to a fasting-like state regardless of changes in food intake. These phenotypes are attributed to the suppression of GH/IGF-1 signaling accompanied by decreased IGF-1 production, but do not involve mTOR signaling, AMPK signaling, and $NAD^+$ metabolism. In the signaling of the endocrine FGF subfamily (FGF15/19, FGF21, and FGF23), β-Klotho on the plasma membrane is required for FGF15/19 and FGF21, and α-Klotho for FGF23. α-Klotho was originally reported as a longevity gene [23], and it inhibits insulin/IGF-1 signaling by its truncated extracellular region circulating in the bloodstream. Thus, it is possible that α-Klotho causes longevity through a similar mechanism to FGF21, but α-Klotho is different from FGF21 in that it causes insulin resistance [22]. FGF21 also decreases preference for sweetness and alcohol via the central nervous system [24–27], which may contribute to the regulation of eating behavior in response to the energy states.

## 7. Regulation of metabolism during fasting by insulin and glucagon

Low blood insulin levels play an important role in the regulation of energy metabolism during fasting. Insulin activates PI3K through phosphorylation of insulin receptor substrate (IRS), and subsequent activation of Akt leads to phosphorylation and translocation of FOXO1 out of the nucleus, resulting in the suppression of the expression of ATGL, the rate-limiting enzyme in lipolysis. In addition, insulin signaling phosphorylates FOXA2 and excludes it from the nucleus, thereby suppressing the expression of HMGCS2, the rate-limiting enzyme for ketone body production. Therefore, the decrease in insulin signaling during fasting is involved in the expression of ATGL, gluconeogenesis-related genes, and ketone

body production-related genes. Fasting also regulates the expression of IRSs and the PI3K activity. The expression of IRS-2 and the PI3K activity are elevated during fasting and decrease immediately after food intake in the liver [28]. In this context, IRS-2 appears to act for a short time after food intake so that it can respond again to the next coming dietary stimuli, whereas the expression of IRS-1 is relatively constant regardless of feeding conditions [28]. Therefore, it is considered that while both IRS-1 and IRS-2 are involved in inhibition of gluconeogenesis immediately after food intake, IRS-1 is mainly involved in glycogen production that is initiated at an interval after that. IRS-1 and IRS-2 differ not only in their expression patterns but also in their functions. IRS-1 deficient mice do not show strong diabetic symptoms, because IRS-2 can compensate for glucose intolerance by promoting the proliferation of pancreatic β cells [29]. The increased transcription of the IRS-2 gene during fasting and its repression after refeeding are regulated by the glucagon receptor-PKA-CREB-regulated transcription coactivator 2 (CRTC2)-CREB pathway and the insulin receptor signaling in the liver, while the regulation mechanism in other tissues remains unclear [30]. Refeeding induces insulin binding to the insulin receptor and downstream PI3K activation, which represses IRS-2 transcription via FOXO1 phosphorylation by Akt.

During fasting, glucagon is secreted from pancreatic α cells in response to hypoglycemia and stimulates glucose production in the liver through gluconeogenesis and glycogenolysis. The glucagon receptor-cAMP-PKA pathway promotes gluconeogenesis by inducing the expression of the catalytic subunits of G6Pase and PEPCK in the liver. PKA-dependent phosphorylation of CREB and dephosphorylation of CRTC2 forms CREB-CRTC2 complex that recruits a histone acetyltransferase, CREB binding protein (CBP) and promotes transcription of the gene encoding PGC-1α. Furthermore, PGC-1α, which is activated by SIRT1-mediated deacetylation and inactivated by general control nonderepressible-5 (GCN5)-mediated acetylation, promotes gluconeogenesis together with FOXO1 and hepatocyte nuclear factor 4α (HNF4α) [30].

## 8. Caloric restriction and life span

Fasting and caloric restriction have received so much attention because of their relationship to longevity. Fasting and caloric restriction to the extent that they do not lead to malnutrition are associated with longevity from nematodes to vertebrates, and are effective against aging and age-related diseases. For example, it has already been known for 80 years that restricting food intake prolongs lifespan in rats and mice [31]. Studies in monkeys showed that feeding a calorie-restricted diet from a young age inhibits the development of obesity, type 2 diabetes, cardiovascular disease, and cancer, and delays the onset of sarcopenia, senile deafness, and brain atrophy [31]. Although the molecular mechanisms by which caloric restriction maintains health and extends lifespan are not yet fully understood, various factors have been identified to be involved, including FGF21, insulin and IGF-1 signaling, mTOR, AMPK, SIRTs, $NAD^+$, FOXO, heat shock factor (HSF), and nuclear factor-erythroid 2-related factor 2 (NRF2) [31].

FOXO is a DNA-binding transcriptional regulator, which promotes the expression of a group of genes involved in DNA repair, autophagy, antioxidant activity, stress tolerance, and cell proliferation [31]. NRF2 and HSF1 are thought to be involved in the maintenance of protein homeostasis and cell structure through induction of antioxidant and drug-metabolizing enzymes, thus extending lifespan [32].

mTOR, first found in bacteria collected from Easter Island soil, is a serine/threonine kinase that is activated by a variety of factors and regulates a wide

range of biological processes. mTOR is activated by insulin and IGF-1 signaling, intracellular nutrients such as amino acids and glucose, and energy states as well as oxygen and stresses to regulate cell proliferation, metabolism, nutrition, and environmental stresses [33]. It was also shown that inhibition of mTOR signaling prolongs lifespan in yeast, *C. elegans*, Drosophila, and mouse, but the mechanism is not fully understood [32, 33]. mTOR forms two distinct protein complexes, mTORC1 and mTORC2, each of which phosphorylates different target substrates. While mTORC1 is strongly inhibited by rapamycin, mTORC2 is less affected [32]. Therefore, much remains to be learned about biological functions of mTORC2.

mTORC1 is activated by growth factors such as IGF-1, stresses, energy states, oxygen, and amino acids [33]. mTORC1 promotes ribosome biosynthesis and protein synthesis in the muscles, and inhibits ketone body production in the liver. In addition, it promotes adipocyte differentiation through activation of sterol regulatory element binding protein 1 (SREBP1) and PPARγ, and increases the number and the size of pancreatic β-cells. Furthermore, mTORC1 suppresses autophagy, enhances glycolytic gene expression, and regulates mitochondrial oxidative metabolism. Suppression of mTOR signaling by caloric restriction, methionine-restricted diet, and rapamycin is thought to be involved in longevity, presumably through the complex regulatory mechanisms described above [32].

mTORC2 is activated by insulin, IGF-1, and leptin through PI3K and other pathways, and then phosphorylates Akt, serum- and glucocorticoid-induced protein kinase 1 (SGK1), and protein kinase C (PKC) family members. Akt and SGK1 further phosphorylate FOXO1, which results in nuclear export of FOXO1 and repression of the expression of gluconeogenesis-related genes [32, 33]. Thus, mTORC2 is involved in the regulation of energy metabolism, but its role in longevity is still unclear.

SIRTs are known as "longevity genes," and forced expression of SIRTs extends lifespan in yeast, *C. elegans*, and Drosophila [34]. SIRTs are $NAD^+$-dependent deacetylases. Since $NAD^+$ and NADH levels are strongly related to fatty acid oxidation, ketone body production, and gluconeogenesis, SIRTs are thought to be involved in lifespan extension by fasting. Although the molecular mechanisms of longevity are not fully understood, it was reported that SIRT1 and AMPK activates PGC-1α, and that overexpression of SIRT1, SIRT3, and SIRT6 results in enhanced genomic stability, suppression of NF- κB signaling, and improvement of metabolic homeostasis via histone deacetylation [31]. In addition, Sir2.1 of *C. elegans* activates DAF-16, a FOXO-type transcription factor [34].

Insulin and IGF-1 signals function as a sensor of nutritional states, and are suppressed by fasting and caloric restriction. During fasting, IGF-1 signaling is repressed by FGF21 as described earlier. On the other hand, the mechanism of suppression of IGF-1 signaling by caloric restriction is complex and may differ among species. In humans, it was reported that protein intake is more important than total caloric intake for the suppression of IGF-1 signaling [31]. Insulin and IGF-1 signals have been shown to be involved in longevity, but its mechanism remains unclear. Genome-wide association studies (GWAS) showed an association between insulin and IGF-1 signaling and human lifespan, and also suggested that Akt, FOXO, PI3K, and SGK signaling pathways are involved in longevity [35]. Treatment of epithelial cells with serum from patients with Laron syndrome, which is characterized by refractoriness to GH, increases the expression of the superoxide dismutase 2 ($SOD_2$) and decreases the expression of mTOR [36]. In addition, FOXO transcription factors are upregulated in the absence of IGF-1 signaling [36]. These studies suggest that insulin and IGF-1 signaling suppresses stress resistance responses. In *C. elegans*, mutations of the daf-2 gene that suppresses the activity of DAF-2, a homolog of the insulin/IGF-1 receptor, increase lifespan through the inhibition of the PI3K-phosphoinositide-dependent

kinase (PDK)-Akt pathway [34]. In Snell mice with mutations in pituitary-specific positive transcription factor-1 (PIT-1) and Ames mice with mutations in the gene encoding the regulator of PIT-1, their lifespan increased by up to 68% compared to control mice [36]. In these mice, in addition to GH, thyroid stimulating hormone (TSH) and prolactin levels were low. However, the observations that lifespan is extended in GH receptor-deficient mice and in mice lacking the gene encoding pregnancy-associated plasma protein A (PAPP-A), an activator of IGF-1 [36], support the idea that insulin and IGF-1 signaling contributes to longevity.

## 9. Energy states and epigenetics

The energy states of organisms, such as fasting and caloric restriction, are memorized in cells, and can lead to the development of future diseases. Recently, epigenetic mechanisms have received much attention in the field of metabolism. The epigenome is a mechanism for regulation of gene expression without changing the genome sequence. Due to its high plasticity, epigenetic regulation is suitable for the memory of metabolic states. Such epigenetic regulations include covalent modifications of DNA, histone, and RNA. More than 100 modifications have been identified, among which methylation, phosphorylation, ubiquitination, and acetylation are well studied, while glycosylation, crotonylation, and succinylation, although functional, are poorly understood [37]. These modifications influence spatial chromatin structure and recruitment of transcription factors and enzymes that are involved in chromatin remodeling. A number of studies have revealed that fasting and obesity-associated diseases that induce a fasting-like metabolic state have a functional link to the epigenome.

Accumulating evidence indicates that diet is one of the most important environmental factors that cause epigenetic changes in the growth process of organisms. One striking evidence of dietary effects on early development was found in honey bees [38]. Female honey bees have two distinct phenotypes, and female larvae that receive royal jelly exclusively develop into fertile queen bees while the other females develop into sterile workers. These phenotypic changes are accompanied by differential DNA methylation patterns and gene expressions between queens and workers through DNA methyltransferase 3 ($DNMT_3$). Such epigenetic alterations during development in response to nutritional states are also found in mammals. A well-known example is the nutritional effect on coat color of the mouse by DNA methylation of the Agouti viable yellow ($A^{vy}$) locus [38]. Insertion of an endogenous retrovirus (ERV) upstream of this gene locus causes constitutive expression of the Agouti gene as the ERV functions as an alternative promoter, resulting in yellow coat color and adult-onset of obesity. The DNA methylation level at the ERV locus increased in offspring but not in the mother in response to gestational intake of compounds related to the methionine cycle including folate. In rats, the locus-specific change in DNA methylation of the Ppara gene was also observed in offspring of the father or mother fed with a low protein diet [38]. It is also reported that feeding high-fat diet to pregnant Japanese macaques led to global hyperacetylation of histone H3 in their offspring [38]. In humans, epidemiological studies developed the important concept of 'Developmental Origins of Health and Disease' (DOHaD), which proposes that unfavorable adaptation to nutritional stress at the embryonic stage is a risk for abnormal growth and development and subsequent health disorders later in life [38]. Cohort studies on the Dutch Famine of 1944–1945 revealed that the severe maternal starvation during the peri-conceptional period induced mental and metabolic abnormalities such as obesity and type 2 diabetes along with changes in DNA methylation in the next generation.

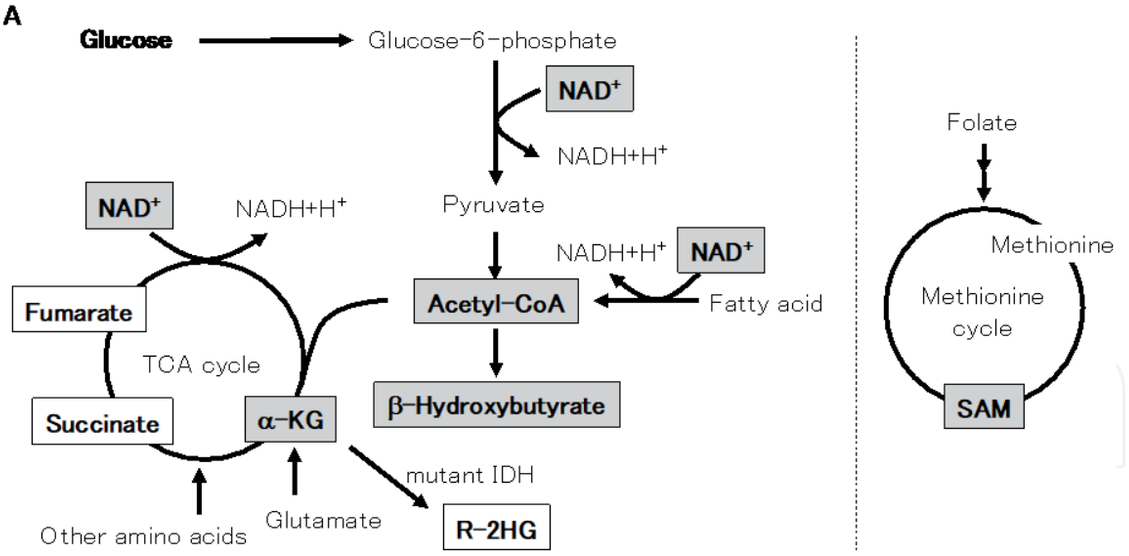
Although epigenetic profiles are more prone to be altered by nutritional states during peri-conceptional, gestational, and early postnatal periods, dietary effects on the epigenetic state are also found in adult animals. Several lines of evidence have revealed that overnutrition induces epigenetic changes in adult organs. One study revealed 232 differentially methylated regions (DMRs) on the genomic DNA in the adipose tissue between mice fed a normal chow diet and those fed a high fat diet [39]. Importantly, the DMRs were also found in humans when comparing lean and obese subjects. Histone modifications are also influenced by diet. It is reported that mice fed a high-fat diet show the increased acetylation levels of histone H3K9 and H3K18 in the genomic regions encoding TNFα and CCL2 in the liver [40]. A mass spectrometry-based approach showed that high-fat diet increased the level of di-methylated histone H3K36 (H3K36me2) and other histone modifications in the mouse liver [41]. In human urine-derived podocyte-like cells, palmitate treatment induced sustained activation of FOXO1 even in the presence of insulin, which was accompanied by the increased H3K36me2 level in the promoter region of the FoxO1 gene [42]. The H3K27me3 in the FoxO1 promoter region decreased in the kidney of rat fed a high-fat diet. In addition to the DNA and histone modifications, RNA methylation is an emerging regulatory mechanism of epigenetics/epitranscriptomics. For example, there reported that a high-fat diet enhanced the expression of fat mass and obesity-associated protein (FTO)/alkB homolog 9 (ALKBH9), an RNA demethylase, and suppressed the N6-methyladenosine (m6A) levels in the mouse adipose tissue, which resulted in obesity [43]. The beneficial effects of fasting on health have also attracted attention in the field of epigenetics. Fasting is implicated in histone modifications via ketone body production as described below. In addition, a clinical human study showed that fasting stress increased methylated CpG sites of the Leptin and Adiponectin genes only in the group born with normal body weight but not in the group born with low body weight [44]. Collectively, epigenetic profiles reflect nutritional states not only in the fetus and infants but also in adults.

## 10. Metabolite and co-factor-mediated regulation of epigenetic enzymes

Although it is not well understood which component in the diets is responsible for epigenetic changes in organisms, it should be noted that the activity of epigenetic enzymes depends on metabolites and co-factors such as metals. These enzymes include acetyltransferases, deacetylases, methyltransferases, and demethylases for DNA, histone, and RNA. In reactions that add or remove epigenetic modifications, these enzymes utilize metabolites derived from metabolic pathways including glycolysis, fatty acid oxidation, the methionine cycle, and the TCA cycle. These metabolic pathways supply metabolites, such as acetyl-CoA, $NAD^+$, S-adenosylmethionine (SAM), and α-KG as substrates to the enzymes (**Figure 7**). Ferrous iron is also an essential co-factor for epigenetic demethylases. Considering that the kinetic and thermodynamic properties of the interaction between an epigenetic enzyme and a metabolite are in a similar range as the physiological concentrations of metabolites [37], it is possible that dynamic changes in the availability of metabolites and co-factors may affect epigenetic outcomes. Here, we discuss the potential for regulation of epigenetic modifications through metabolites and co-factors.

HATs, such as CBP/p300, are enzymes that transfer an acetyl group from acetyl-CoA to lysine residues on histone proteins (**Figure 7B**). Acetyl-CoA is supplied from various nutrients through metabolic pathways such as glycolysis, the TCA cycle, and fatty acid oxidation (**Figure 7A**). In a study examining which nutrient-derived acetyl-CoA alters histone acetylation, only lipids, among various nutrients, induced direct acetylation of histones via fatty acid oxidation in mammalian cells [45]. Another

A




B

| Epigenetic enzymes | Substrates and co-factors | Inhibitory metabolites |
|---|---|---|
| **Histone acetyltransferase** | Acetyl-CoA | |
| **Histone deacetylase (SIRTs)** | $NAD^+$ | Butylate, β-hydroxybutyrate |
| **Methyltransferase** | SAM | |
| **Demethylase (2OGDDs)** | $O_2$, α-KG, $Fe^{2+}$ | R-2HG, succinate, fumarate |

**Figure 7.**
*Metabolite-mediated regulation of epigenetic enzymes. A. Production of substrates and co-factors of epigenetic enzymes in the metabolic process, B. Substrates, co-factors, and inhibitory metabolites of epigenetic enzymes.*

study showed that the treatment of pancreatic β cells with palmitate increased HAT activity and histone acetylation [44]. However, a mass spectrometry-based study demonstrated that a high-fat diet rather decreased acetyl-CoA levels in the mouse white adipose tissue, which correlated with histone acetylation [45], suggesting that the regulation of histone acetylation by metabolites is more complex in obesity.

Acetyl groups on histones are removed by the action of HDACs. SIRTs (SIRT1–7), a class III HDACs, are $NAD^+$-dependent deacetylases that sense the energy state in cells (**Figure 7**). They have been shown to be involved in longevity in a variety of species, including yeast, *C. elegans*, and Drosophila, although epigenetic mechanisms are not fully understood [32, 33]. Importantly, a decrease in $NAD^+$ levels due to activated glycolysis was sufficient to inhibit the activity of $NAD^+$-dependent deacetylases and promoted histone H4K16 acetylation during differentiation of murine muscle cells [37], suggesting that fasting can be involved in the regulation of epigenetic enzymes. Butylate, a short-chain fatty acid produced by intestinal fermentation, is known to inhibit histone deacetylases [45]. Butyrate and a class I HDAC inhibitor have been reported to suppress obesity-associated phenotype in a mouse model of high-fat diet induced obesity (**Figure 7B**) [45]. Similarly, β-hydroxybutyrate, one of the ketone bodies, inhibits class I HDACs (HDAC1, 2, 3, and 8) (**Figure 7B**) [6]. Considering that the inhibition potency (IC50) of β-hydroxybutyrate on these HDACs is around 2–5 mM in an in vitro assay, and that the concentration of β-hydroxybutyrate in humans during long-term fasting is 6–8 mM, β-hydroxybutyrate is a potential physiological inhibitor of HDACs [6]. In the mouse kidney, fasting induced hyperacetylation of histone H3K9 and H3K14, and the HDAC-mediated expression of FoxO3 enhanced the expression of oxidative stress resistance genes [46].

DNMTs, histone methyltransferases (e.g., enhancer of zeste homolog 2 (EZH2), SET domain-containing methyltransferases (SETs), and mixed-lineage leukemias

(MLLs)), and RNA methyltransferase (e.g., methyltransferase like 3 (METTL3) and METTL14) require SAM as a methyl donor for methylation of DNA, histone, and RNA, respectively (**Figure 7B**). SAM is provided by the methionine cycle from dietary components such as methionine and folate (**Figure 7A**). Decreased folate levels in the circulation was reported in patients with type 2 diabetes, and the folate levels were correlated with DNA methylation levels in the liver [44]. Additionally, administration of folate to mice fed a high-fat diet altered the DNA methylation patterns of genes in the adipose tissue and improved obesity-associated phenotype [44].

Among epigenetic demethylases, DNA demethylases (ten-eleven translocation methylcytosine dioxygenases (TETs)), histone lysine demethylases with a JmjC domain (e.g., KDMs), and RNA demethylases (ALKBH5 and FTO/ALKBH9 require oxygen and α-KG as substrates and ferrous iron as a cofactor (**Figure 7B**). α-KG is an intermediate metabolite of the TCA cycle and is also supplied by a flux of amino acids such as glutamate (**Figure 7A**), while ferrous iron is taken up from outside the cell via transferrin receptors or supplied internally by ferritin-selective autophagy [47]. Interestingly, these demethylases are classified into the 2-oxoglutarate-dependent dioxygenase (2OGD) family, and the structure of their catalytic domain is highly conserved among all the enzymes [48]. Notably, the iron-binding site of the enzymes is composed of a highly conserved amino acid sequence, that is, histidine, and aspartate or glutamate located two amino acids away from the histidine, followed by histidine located around a hundred amino acids away from the two amino acids (HXD/G...H). A ferrous iron molecule bound to these amino acids serve as a catalytic center of the demethylase, which oxidizes the methyl group of the substrates and removes the methyl group from DNA, histone, and RNA. Several studies suggested that both α-KG and iron are critical regulators of 2OGDs. α-KG has been shown to increase during adipocyte differentiation and to promote differentiation through demethylation of H3K9 at the Pparg locus [49]. Similarly, adipocyte differentiation in 3T3-L1 cells, which involves dynamic changes in the epigenome, is inhibited by iron depletion, although how the ferrous iron level is altered during differentiation has not been explored [50, 51]. It is also noteworthy that some types of cancer cells harboring mutations on IDH1 and IDH2 produce an inhibitor of 2OGDs, (R)-2-hydroxyglutarate (R-2HG) (**Figure 7**) and show characteristic DNA and histone hypermethylation [37]. Additionally, fumarate and succinate can inhibit 2OGDs (**Figure 7**), and deletion of fumarate dehydrogenase and succinate dehydrogenase induced histone and DNA hypermethylation [37]. Considering the recent findings that KDM5A/JARID1A and KDM6A/UTX function as oxygen sensors whose demethylase activity is inhibited under hypoxia [52, 53], it is possible that change in the concentrations of metabolites and cofactors may also affect the demethylase activity of 2OGDs, and thus control epigenetic consequences of cellular processes.

Therefore, it is conceivable that energy states could regulate epigenetic mechanisms and be memorized, subsequently influencing the onset of a variety of diseases. Thus, understanding the relationship between energy states and the epigenome is essential for establishment of an appropriate diet-based therapy. As future challenges, it is necessary to elucidate how concentrations of metabolites and cofactors change during biological processes, and which epigenetic enzymes are responsible for the metabolite- and/or cofactor-mediated epigenetic alterations. However, measuring local concentrations of metabolites and cofactors, especially in the nucleus, has been difficult due to technical barriers. We have recently developed a fluorescence resonance energy transfer (FRET)-based biosensor to measure nuclear α-KG concentrations and have found that nuclear α-KG concentrations increase with adipocyte differentiation [54]. The development of such tools will shed light on the regulatory mechanisms of the epigenome by biomolecules in the future.

## 11. Future perspectives

Now that the development of the internet and other factors have made it easy for non-medical professionals to obtain medical knowledge, the effects of caloric restriction, fasting, the order in which foods are eaten, and the nutritional composition of diets have become familiar topics of interest in the treatment of diabetes and obesity. Therefore, it is more and more important than ever for researchers to understand whole body metabolism based on accurate evidence. There is no doubt that diet plays an important role in the maintenance of health, and new molecular mechanisms including epigenetics have recently emerged in addition to the widely accepted concepts on metabolism. In addition, the involvement of unknown regulatory factors has been implicated. Extensive research will lead to a better understanding of energy metabolism in the body and contribute to the extension of healthy life.

## Conflict of interest

None.

## Author details

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