

Reassessing triglyceride synthesis in adipose tissue

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The synthesis and breakdown of triglycerides in adipose tissue and muscle is a crucial element of energy metabolism because it ensures that adequate fuel is available during starvation. Triglyceride turnover determines the availability of fatty acids for utilization by mammalian tissues, and any dysfunction in this process can lead to alterations in glucose metabolism, insulin resistance and type 2 diabetes. Our understanding of the reactions involved in triglyceride synthesis is currently being reassessed, primarily because of the recently identified role that re-esterification of fatty acids plays in triglyceride deposition and, thus, in controlling fatty-acid availability. Here, we review recent information on triglyceride synthesis and introduce the pathway of glyceroneogenesis as an important and highly regulated source of glyceride-glycerol in adipose tissue.

Triglyceride synthesis

The textbook view of triglyceride synthesis is that three molecules of fatty acyl CoA are esterified to one molecule of glycerol-3-phosphate (G-3-P), which is derived from glucose via glycolysis, to form a molecule of triglyceride. The overall reaction sequence for triglyceride synthesis is of more than esoteric interest because the synthesis and deposition of triglyceride in adipose tissue and muscle is a major factor in regulating energy metabolism in mammals. It is generally held that insulin controls triglyceride synthesis in adipose tissue and muscle by increasing glucose uptake and regulating its conversion to G-3-P via glycolysis. Glucose metabolism via glycolysis generates dihydroxyacetone phosphate, which can be reduced to G-3-P for triglyceride synthesis in these tissues. However, there is increasing evidence that the pathway outlined above is not the major source of G-3-P for triglyceride synthesis in mammals. In fact, a metabolic pathway termed glyceroneogenesis (i.e. the synthesis of glyceride-glycerol from sources other than glycerol and glucose) is emerging as an important source of carbon for glyceride-glycerol in mammals, both during starvation and after ingestion of a diet high in carbohydrates.

Glyceroneogenesis

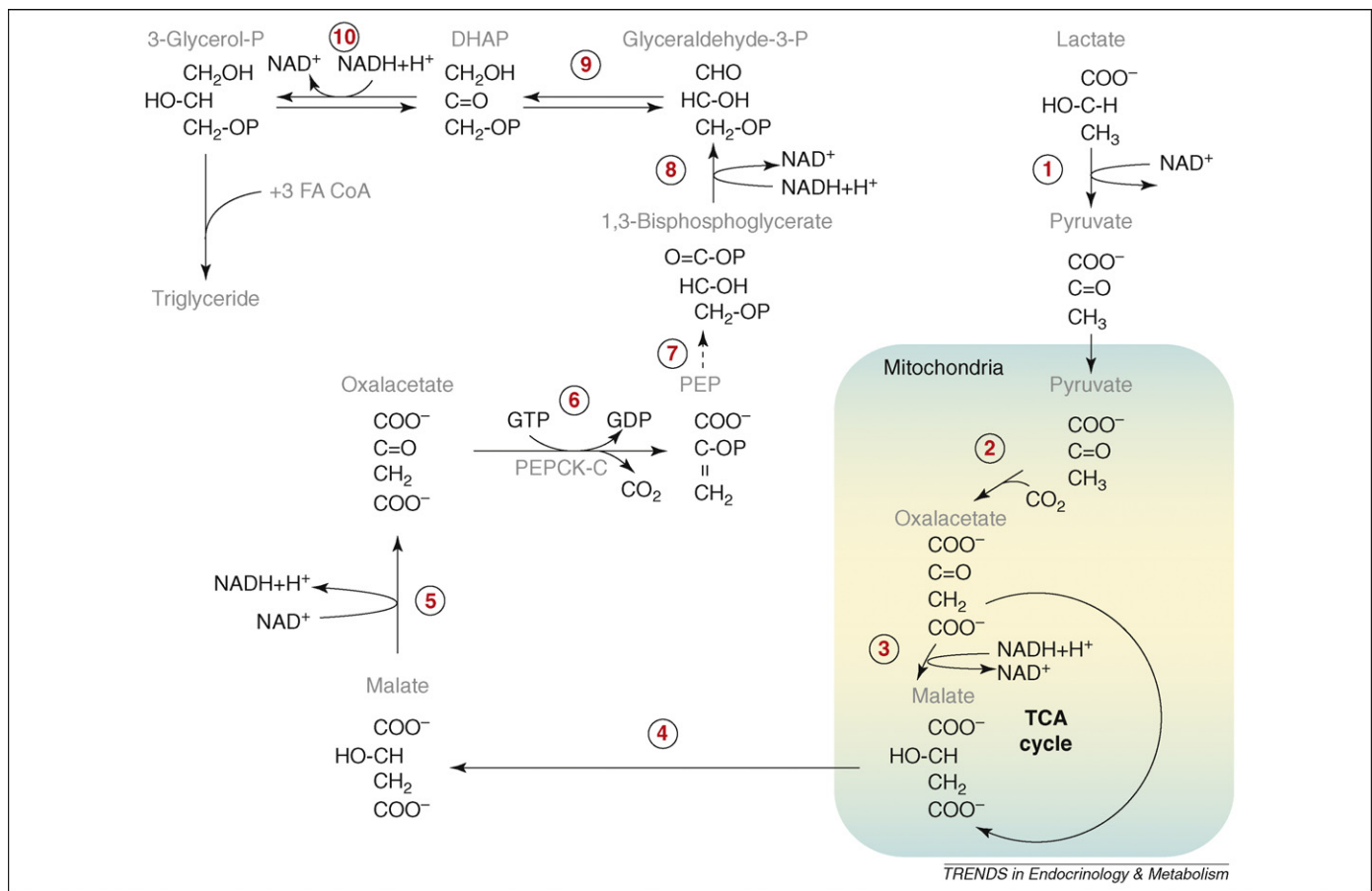
The discovery of glyceroneogenesis was spurred by the initial finding that adipose tissue contains both pyruvate carboxylase and the cytosolic isoform of phosphoenolpyruvate carboxykinase (GTP) (PEPCK-C) [1]. There is also a

mitochondrial isoform of PEPCK, PEPCK-M, the metabolic role of which is less well defined. Both PEPCK isoforms are present in the genome of all eukaryotic species studied to date, so it is likely that both enzymes have a crucial role in metabolism. Interestingly, species vary in the relative amounts of the two forms of PEPCK found in tissues, including the liver, kidney cortex, and brown and white adipose tissue. Humans have 50% of each form in these tissues, whereas rodents such as the rat and the mouse (the most widely studied experimental animals) have 90% PEPCK-C. Studies on the metabolic role of PEPCK-M have largely focused on birds, where it comprises 100% of the total activity of PEPCK in the liver. A detailed description of the proposed metabolic role of PEPCK-M can be found elsewhere [2,3].

PEPCK-C catalyzes the conversion of oxalacetate, a citric acid cycle intermediate, to PEP and is a key step in both hepatic and renal gluconeogenesis. PEPCK-C was generally considered exclusively a gluconeogenic enzyme. This exclusivity changed in 1967 when Ballard *et al.* reported the incorporation of [¹⁴C]-labeled pyruvate into glyceride-glycerol of triglyceride in epididymal adipose tissue isolated from fasted rats and suggested that an abbreviated version of gluconeogenesis, subsequently termed glyceroneogenesis [4], was responsible [1]. Further work (described in Refs [5,6]) formed the basis for the proposal that during starvation, adipose tissue synthesizes triglycerides from G-3-P that is generated via glyceroneogenesis and not from G-3-P generated from glucose via glycolysis. After these studies, White *et al.* [7], using specifically labeled [¹⁴C]pyruvate, observed a labeling pattern in glyceride-glycerol isolated from the triglyceride of rat epididymal adipose tissue that was consistent with incorporation of pyruvate carbon via glyceroneogenesis. Adipose tissue normally has negligible levels of glycerol kinase, so glycerol cannot be directly phosphorylated to G-3-P and end up as glyceride-glycerol. There is also very little glycogen in this tissue, negating glycogen as a major source of glucose available for the synthesis of G-3-P used for triglyceride synthesis. The metabolic significance of glyceroneogenesis is that any compound that can enter the citric acid cycle and form oxalacetate can contribute to triglyceride synthesis (i.e. this process does not depend on a supply of glucose) (Figure 1).

Glyceroneogenesis and the triglyceride–fatty-acid cycle
In 1962, Martha Vaughan first pointed out that in the absence of added glucose, adipose tissue *in vitro* re-esterifies ~30% of the free fatty acid (FFA) released from

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Figure 1. The pathway of glyceroneogenesis in mammalian tissues. The pathway of lactate and pyruvate conversion to glyceride-glycerol in mammalian tissues is presented (from right to left), with specific regulatory steps numbered. This pathway is initiated by the carboxylation of pyruvate to oxalacetate and its entry into the citric acid cycle (2), where it is reduced to malate by NAD malate dehydrogenase (3). Malate leaves the mitochondria on the dicarboxylate shuttle (4) where it is reoxidized to oxalacetate by the cytosolic form of NAD malate dehydrogenase (5). This process produces NADH in the cytosol for the future reductive synthesis of 3-glycerol phosphate. The oxalacetate is decarboxylated to phosphoenolpyruvate by PEPCK-C (6) and converted via reversal of glycolysis to 1,3-bisphosphoglycerate (7), which is subsequently oxidized to glyceraldehyde-3-phosphate by glyceraldehyde-3-phosphate dehydrogenase (8). The latter reaction uses the NADH generated by the oxidation of malate in the cytosol (see step 5). Glyceraldehyde-3-phosphate is in equilibrium with dihydroxyacetone phosphate via an isomerase (9); it is then reduced to 3-glycerol phosphate via 3-glycerol phosphate dehydrogenase, which requires a second molecule of NADH. Note that when lactate is the substrate for glyceroneogenesis, the molecule of NADH, which is required for 3-glycerol phosphate dehydrogenase, is formed by lactate dehydrogenase (1). The carbon skeletons of amino acids also enter the citric acid cycle and can contribute to the synthesis of glyceride-glycerol via glyceroneogenesis by being converted to malate in the mitochondria and, subsequently, following the pathway outlined in the Figure. The potential source(s) of the second NADH that is required for glyceride-glycerol synthesis when lactate is not the substrate is discussed in detail in the text. It is most likely generated by a reversal of the malate-aspartate shuttle that effectively moves NADH from the mitochondria to the cytosol.

triglyceride during lipolysis [8]. This seemingly wasteful form of ‘futile cycling’ was later termed the triglyceride–fatty-acid cycle to describe a process in which the FFAs generated by lipolysis are esterified back to triglyceride [9]. As an example, Jensen *et al.* reported that ~60% of the FFA formed during lipolysis in a 60 h starved human is recycled back to triglyceride in tissues such as adipose tissue, liver and muscle [10]. Such an extensive rate of recycling indicates that lipolysis results in the release of more fatty acids than are needed for oxidative metabolism, with the excess being esterified back to triglyceride. The high degree of triglyceride cycling most likely reflects the fact that lipolysis is not sensitive enough to provide the exact amount of fatty acids required for energy metabolism and, thus, generates more than is required, recycling the unused fatty acids back to triglyceride. The re-esterification process requires energy and costs ~3% of the total energetic content of a molecule of triglyceride; this is the apparent energetic price we pay for having an ample quantity of fatty acids available to support energy metabolism.

One potential metabolic risk to the extensive triglyceride–fatty-acid cycling is that an excess of fatty acids interferes with glucose uptake from the blood [11] and glucose metabolism in skeletal muscle [12], resulting in insulin resistance and ultimately, type 2 diabetes [13]. Denis McGarry [14] suggested that the rate of fatty-acid re-esterification in tissues that store triglyceride, such as adipose tissue, is crucial for controlling diabetes. It is noteworthy that the anti-diabetic drug rosiglitazone, which is a PPAR γ 2 agonist, induces transcription of the gene for PEPCK-C in adipose tissue [15] and increases the rate of glyceroneogenesis in this tissue as well [16]. In fact, glyceroneogenesis has been proposed to be a major target of rosiglitazone in the correction of dyslipidemias in humans [17–19]. Rosiglitazone also enhanced glyceroneogenesis in adipose tissue explants from human subjects [17], determined by the incorporation of [1- 14 C]pyruvate into glyceride-glycerol after three days of drug exposure [18]. In addition, rosiglitazone increased the expression of the gene for glycerol kinase [20], and the activation of PPAR γ 2 induces transcription of the gene for G-3-P dehydrogenase

in adipose tissue [21]. Thus, both the direct phosphorylation of glycerol via glycerol kinase and the generation of G-3-P by glyceroneogenesis stimulate triglyceride synthesis in adipose tissue and contribute to the correction of dyslipidemia. The FFA released during lipolysis will also activate PPAR γ 2 and induce transcription of the gene for PEPCK-C, further increasing the rates of FFA re-esterification. Thus, as the concentration of FFA in the blood rises, processes involved in its re-esterification back to triglyceride are activated, and FFAs subsequently are removed from circulation and stored in adipose tissue.

Determining the relative contribution of glyceroneogenesis versus glycolysis in adipose tissue *in vivo*

Few *in vivo* studies have measured and compared the relative formation of glyceride-glycerol from glucose via glycolysis to that formed via glyceroneogenesis. In one study, Botion *et al.* quantified the relative contribution of glyceroneogenesis and glycolysis to glyceride-glycerol synthesis *in vitro* and *in vivo* in the rat by using [$^3\text{H}_2\text{O}$] and U-[^{14}C]glucose as tracers [22]. Rats were adapted to a carbohydrate-free, high-protein diet, which induced PEPCK-C in white adipose tissue. Pyruvate incorporation into glyceride-glycerol of the triglyceride in adipose tissue *in vitro* increased, but only in the absence of glucose. Two aspects of these experiments deserve further consideration. First, glyceroneogenesis was measured by subtracting the rate of incorporation of [^{14}C] of U-[^{14}C]glucose from the rate of incorporation of [^3H] into glyceride-glycerol. Because this calculation subtracts the [^{14}C] incorporated into glyceride-glycerol via [^{14}C]lactate, it potentially underestimates the rate of glyceroneogenesis and overestimates the glycolytic contribution. Second, feeding the animals a carbohydrate-free, high-protein diet increased PEPCK-C activity and did not affect the rate of glyceroneogenesis, indicating that glyceroneogenesis is regulated by other factors, such as substrate flux over the pathway or the availability of specific precursors.

Chen *et al.* used $^2\text{H}_2\text{O}$ as a tracer, together with mass isotopomer analysis, to determine the relative contribution of glyceroneogenesis and glucose (via glycolysis) to G-3-P synthesis in adipose tissue and liver of mice. These experiments used extended dietary treatments, in which mice were fed either a high- or a low-carbohydrate diet, or treated with rosiglitazone for 75 days, and the rate of glyceroneogenesis was determined in the adipose tissue of the animals. Glyceroneogenesis in adipose tissue increased from 17% in mice fed a high-carbohydrate diet to 50% in mice fed a low-carbohydrate diet. In addition, rosiglitazone-induced glyceroneogenesis increased from 17% to 53%. The authors concluded that 'glyceroneogenesis may play a central role in the antilipolytic actions of thiazolidiones on adipose tissue' [23].

Finally, Nye *et al.* analyzed the effect of 48 h of fasting and five days of sucrose feeding on the relative contribution of glyceroneogenesis and glucose via glycolysis to the synthesis of glyceride-glycerol in the rat [24]. The rate of glyceroneogenesis was quantified using the tritium ($^3\text{H}_2\text{O}$) labeling of body water method [25], and the contribution of glucose was determined using [U- ^{14}C]glucose

tracer [24]. In epididymal and mesenteric fat of chow-fed (control) rats, glyceroneogenesis accounted for ~90% of triglyceride glycerol synthesis. Fasting did not alter glyceroneogenesis in adipose tissue, and the contribution of glucose was negligible. In response to sucrose feeding, glyceride-glycerol synthesis via both glyceroneogenesis and glycolysis nearly doubled relative to control animals; however, glyceroneogenesis remained quantitatively higher than glucose (via glycolysis) as a source of G-3-P. Enhancement of triglyceride-fatty-acid cycling by epinephrine infusion stimulated glyceroneogenesis in adipose tissue, whereas the contribution of glucose to glyceride-glycerol synthesis was not detectable under these conditions.

Estimates of the relative contribution of glyceroneogenesis and glycolysis to glyceride-glycerol varied in the three rodent studies described above. It is likely that technical differences accounted for such variations. For example, Botion *et al.* [22] did not correct for the incorporation of [^{14}C] from lactate, whereas Nye *et al.* [24] found that accounting for the contribution of lactate decreased the contribution of glycolysis to glyceride-glycerol formation. However, all three studies agreed that fasting increases glyceroneogenesis in adipose tissue relative to glycolysis. The most surprising finding was made by Nye *et al.* [24], who noted that glyceroneogenesis was the major pathway of glyceride-glycerol synthesis in adipose tissue and skeletal muscle in rats fed a diet supplemented with sucrose for five days, when rates of lipogenesis were markedly elevated. This was unexpected because the rate of glucose conversion to fatty acids was very high under these dietary conditions; therefore, one would predict a robust rate of glucose metabolism via glycolysis to support lipogenesis. However, the fact that glucose is not the major precursor of glyceride-glycerol in triglyceride from adipose tissue indicates that both glyceroneogenesis and glycolysis are occurring in this tissue simultaneously. These results differ from those from mouse studies performed by Chen *et al.* [23], which found that glucose was the predominant source of glyceride-glycerol in both visceral and epididymal adipose tissue in response to high-carbohydrate-diet feeding for 75 days. It is possible that the differences in the relative contribution of glyceroneogenesis and glucose via glycolysis noted in the two reports are due to variations in the time the animals were fed the high-carbohydrate diet and different labeling procedures used by Chen *et al.* [23].

Glyceroneogenesis also occurs in the liver

The rate of hepatic glyceroneogenesis in humans was determined in two studies by Kalhan and colleagues [25,26]. In the first, the contribution of pyruvate to the glycerol of triglyceride was compared between pregnant and non-pregnant women by using $^2\text{H}_2\text{O}$ labeling of body water. After a 16 h fast, ~10–60% of glycerol was derived from pyruvate via glyceroneogenesis, indicating that hepatic glyceroneogenesis from pyruvate was a major contributor to plasma triglyceride synthesis [25]. In the second study, the contribution of glyceroneogenesis to VLDL triglycerides in the blood of subjects with type 2 diabetes mellitus was determined using the same $^2\text{H}_2\text{O}$ labeling of body water technique [25]. Subjects with type 2 diabetes

were studied before and after a six-month behavioral intervention therapy, involving increased exercise and dietary modifications. At each visit, a hyperinsulinemic-normoglycemic clamp was administered to quantify insulin sensitivity and examine the effect of insulin on glyceroneogenesis. Glyceroneogenesis contributed ~54% to VLDL glyceride-glycerol in type 2 diabetes, compared with the ~12% contribution of plasma glucose. The infusion of insulin plus glucose during the hyperinsulinemic clamp had no effect on glyceroneogenesis, even when insulin sensitivity had improved after clinical intervention. These results indicate that glyceroneogenesis, and not glycolysis, is the predominant source of glyceride-glycerol carbon for VLDL triglycerides in normal healthy subjects and in subjects with type 2 diabetes, and that this process is not affected by a short (4 h) infusion of insulin and glucose.

What is the carbon source for glyceroneogenesis?

As mentioned previously, studies by Nye *et al.* indicate that both glycolysis and glyceroneogenesis occur simultaneously in adipose tissue of rats fed a lipogenic diet [24]. By analogy with gluconeogenesis, we assume that any compound that can enter the citric acid cycle as a four- or five-carbon intermediate can generate the G-3-P needed for triglyceride synthesis. This, however, requires PEPCK activity in tissues that make triglyceride and an available source of citric acid cycle anions. For example, the carbon skeletons of amino acids (e.g. alanine, glutamine, asparagine and aspartate) could be likely carbon source candidates for glyceroneogenesis, especially in the liver, a tissue that actively metabolizes these amino acids. Of course, both lactate and pyruvate are important potential sources of G-3-P.

The arteriovenous gradients across adipose tissue in humans indicate a net uptake of glucose and a release of lactate. Interstitial levels of lactate in adipose tissue are substantially higher than those in the plasma and are even higher in obese, as compared with lean, subjects [27]. This suggests that adipose tissue utilizes glucose to produce lactate and G-3-P to generate triglycerides. However, Nye *et al.* reported that pyruvate is the predominant carbon source for G-3-P for glyceride-glycerol even in the presence of increased glucose uptake by adipose tissue [24]. One explanation for these differences is that adipose tissue includes a mixture of cell types (i.e. in addition to adipocytes, it contains endothelial cells and macrophages that primarily metabolize glucose to lactate) [28,29]. Mature adipocytes are fully differentiated cells that exhibit an adipocyte-like phenotype and store triglyceride. Preadipocytes, which are also present, do not deposit triglyceride [30]. Therefore, each of these cell types might exhibit different patterns of substrate utilization depending on the hormonal environment and levels of nutrients. By comparison, the liver also carries out both glycolysis and gluconeogenesis by having glycolytic hepatocytes in the pericentral region and gluconeogenic hepatocytes in the periportal region. Because adipose tissue is composed of multiple cell types, it is possible that glyceroneogenesis and glycolysis occur simultaneously in adipose tissue, but in different cells. In this model, the glycolytic cells would metabolize glucose to lactate, which would then be released into the interstitium to be available as a glyceroneogenic substrate for the triglyceride-storing cell type (Figure 2). The differentiated (i.e. mature) adipocytes, which express PEPCK-C, might rely predominantly on glyceroneogenesis for the deposition of triglyceride. In fact, *in vivo* studies

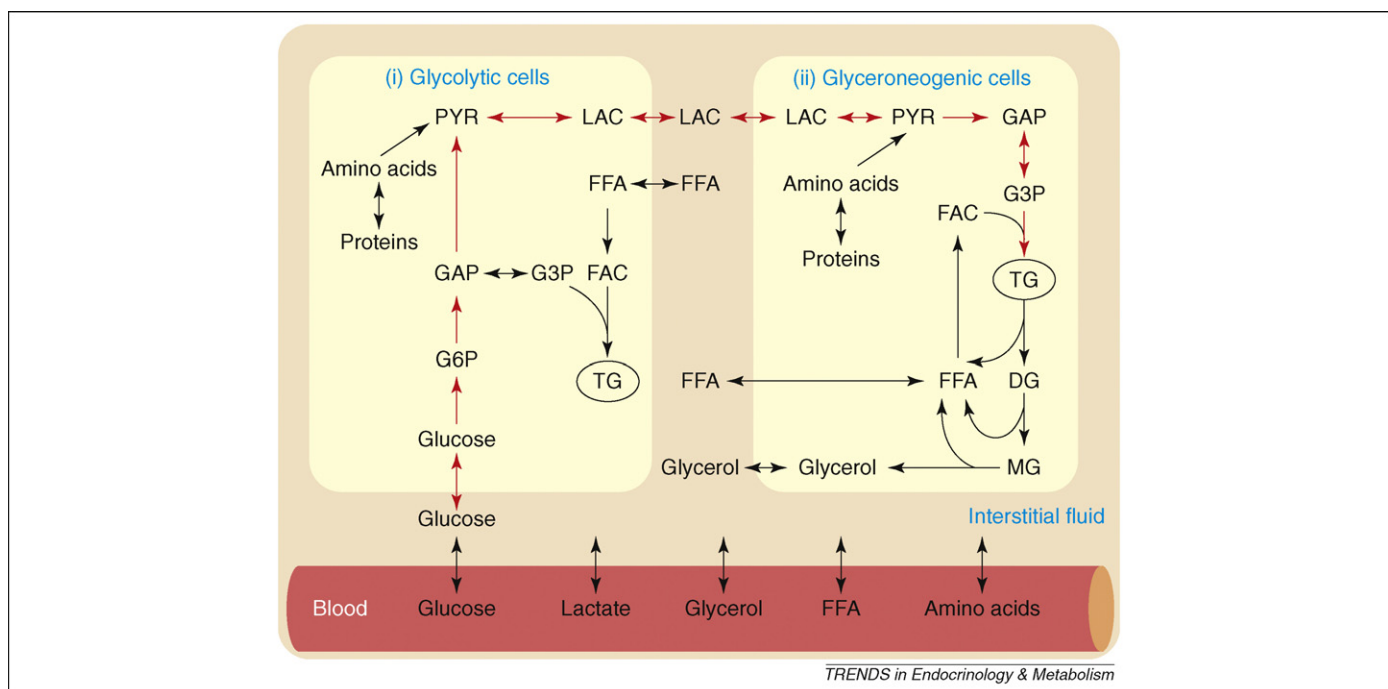


Figure 2. The two-cell hypothesis for the regulation of triglyceride (TG) synthesis in adipose tissue. The hypothesis assumes the presence of two metabolically distinct compartments or cell types in adipose tissue. (i) One cell type (glycolytic) contains minimal triglyceride. (ii) A second cell type (glyceroneogenic) that is a mature adipocyte synthesizes TG and contains PEPCK and the enzymes required for glyceroneogenesis. The two-cell hypothesis also suggests that lactate, produced by glycolysis in the glycolytic cell, is used by the mature adipocytes to form G-3-P via glyceroneogenesis. In addition, amino acids produced by protein turnover in the adipose tissue or taken up from the blood by the tissue can be converted to G-3-P for triglyceride synthesis.

using microdialysis in healthy, lean humans show that after an overnight fast, the interstitial concentration of lactate is considerably higher in abdominal and femoral subcutaneous adipose tissue than in plasma, indicating a local release of lactate. Adipose tissue also expresses the monocarboxylate transporter-1, which might be responsible for lactate release, as well as uptake from the interstitium of adipose tissue, providing further evidence that specific cell types of adipose tissue are capable of carrying out glyceroneogenesis.

Computational analysis of adipose tissue metabolism

The crucial role of glyceroneogenesis in the regulation of triglyceride synthesis was confirmed in a computational model of adipose tissue metabolism in humans that was developed by Kim *et al.* [31]. Using this model, these authors examined the source of carbons for the synthesis of G-3-P in response to various physiological perturbations [31]. The model simulations showed that in order for the simultaneous occurrence of glycolytic and glyceroneogenic contribution to G-3-P, two non-mixing functional pools of triose phosphates were required (Figure 2). These two pools could be either in the same cell or in different cells. The source of carbons for G-3-P, in response to epinephrine, was examined as an example; the simulations showed that the high rate of lipolysis induced by epinephrine was accompanied by a high rate of re-esterification of the fatty acid released. The dominant source of carbons for the G-3-P requirement for triglyceride synthesis was via glyceroneogenesis and not glycolysis. In addition, their data indicated that a high rate of lipolysis accompanied by elevated levels of fatty acyl-CoA increased β -oxidation, and high levels of acetyl CoA resulted in inhibition of oxidation of pyruvate by suppressing PDH, making more pyruvate available for glyceroneogenesis. Taken together, these *in silico* simulations underscore the important role of glyceroneogenesis in glyceride-glycerol synthesis in the adipose tissue.

Conclusions: the potential role of glyceroneogenesis in diabetes

There is a growing body of research that supports the crucial role of glyceroneogenesis in controlling the rate of FFA esterification in mammals; of special importance is the impact that this pathway has on the development of type 2 diabetes in humans. The regulation of FFA recycling back to triglyceride in mammalian tissues (especially, but not restricted to, adipose tissue) via the triglyceride-fatty-acid cycle is important in controlling the concentration of FFA in the blood. It is well established that fatty-acid oxidation spares glucose utilization in tissues such as muscle and liver. This is an important factor in survival because most mammals, including humans, have relatively small stores of carbohydrates available for the metabolism of tissues that require glucose, such as the brain and red blood cells. Thus, in the immediate post-absorptive period, glucose utilization is markedly diminished in favor of fatty-acid oxidation, an effect termed the 'glucose-sparing action of fatty acids.' Although this glucose-sparing effect has clear physiological advantages, it also has some readily apparent drawbacks in diseases such as obesity and type 2 diabetes, in which lipid and carbo-

hydrate metabolism are out of balance. As an example, it has been estimated that the concentration of FFA in the blood of an obese human after an overnight fast is 3–4 times higher than that of a non-obese individual (0.2 versus 0.8 mM); in individuals with type 2 diabetes, FFA levels can rise as high as 3.3 mM in the blood [13]. An elevated concentration of FFA inhibits glucose uptake and its oxidation by skeletal muscle, thus increasing the level of fasting blood glucose and inducing insulin secretion [32,33]; this ultimately leads to insulin resistance and often to type 2 diabetes. In this review, we present evidence that glyceroneogenesis is the primary pathway for triglyceride synthesis in adipose tissue and liver, and that the regulation of glyceroneogenesis by hormones and diet is a key element in controlling lipid metabolism in mammals. Considering its central position in regulating triglyceride metabolism, glyceroneogenesis is a timely target for the development of drugs aimed at controlling diabetes.

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