Minireview

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Glyceroneogenesis and the Triglyceride/Fatty Acid Cycle*

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During fasting in all mammals, triglyceride stored in adipose tissue is hydrolyzed by a hormone-sensitive lipase to produce free fatty acids (FFA)[1] and glycerol. Detailed studies of the balance of glycerol and FFA released from white adipose tissue (WAT) during starvation have noted considerable re-esterification of the FFA in adipose tissue during periods of active lipolysis. For example, in rats fasted for 24 h, about 30% of the FFA is recycled back to triglyceride in WAT (1). In humans, the recycling in this tissue has been estimated to be as high as 40% (2). The recycling of FFA also occurs in the liver as part of a triglyceride/fatty acid cycle that accounts for a considerable quantity of fatty acid recycling. Thus the triglyceride/fatty acid cycle includes local intracellular cycling within the adipose tissue and extracellular or systemic recycling, *i.e.* the formation of triglycerides in the liver and possibly skeletal muscle (Fig. 1). Almost 30 years ago, Newsholme and Crabtree (3) discussed the importance of this cycle in metabolic regulation and heat production. Quantitative estimates of the triglyceride/fatty acid cycle in human adults and newborn infants and studies in animals show that only a small fraction of the FFA released as a result of lipolysis in the WAT are oxidized, and the majority are re-esterified to triglycerides in various tissues (2–9). The quantitative estimates of triglyceride/fatty acid cycling vary in different studies in humans, depending upon the methodology employed. Intracellular recycling (primarily fatty acid re-esterification in WAT) appears to represent ~20–30% of the total, whereas non-adipose tissue recycling (primarily hepatic) accounts for ~50% of re-esterification of fatty acids in healthy adults after an overnight fast (Table I). It is important to note that the fraction of FFA released (lipolysis) that is recycled back to triglyceride remains relatively constant (~75%), despite marked changes in the rate of total triglyceride/fatty acid cycling during different metabolic states (Table I). The metabolic significance of this fixed fractional rate of triglyceride/fatty acid recycling remains to be determined. However, it is clear that triglyceride/fatty acid recycling requires the constant generation of glycerol 3-phosphate for triglyceride synthesis, particularly in situations when cycling is increased.

Quantitative changes in the triglyceride/fatty acid cycle have been related to the increased thermogenesis after burns in humans (4), the increased metabolic rate of cachectic patients with esophageal cancer (5), and increased oxygen consumption following leptin administration (6),and to the amplification of substrate flux during acute exercise (7). In addition, triglyceride/fatty acid cycle flux is markedly increased following an 87-h fast in humans (8). Data from studies using healthy human newborn infants have also shown that 75% of the fatty acids released by lipolysis are recycled back to triglycerides (9). In undernourished, intrauterine growth-retarded infants, higher rates of fatty acid oxidation were associated with an increased rate of lipolysis and fatty acid cycling, so that the magnitude of recycling (~76%) was similar to that in normal infants.

What is the metabolic source of glyceride-glycerol needed to support the triglyceride/fatty acid cycle? There are three major possibilities: glucose, via glycolysis, glycerol after phosphorylation by glycerol kinase, or the conversion of pyruvate to glyceride-glycerol via a pathway to be discussed, termed *glyceroneogenesis*.

The Role of Glyceroneogenesis in WAT

More than 30 years ago we (10, 11) and others (12) described a pathway for the re-esterification of FFA during fasting in WAT that involved the generation of 3-glycerol phosphate from precursors other than glucose. This pathway, termed glyceroneogenesis, is an abbreviated version of gluconeogenesis. Glyceroneogenesis is defined as the conversion of precursors other than glycerol or glucose to 3-glycerol phosphate for the synthesis of glyceride-glycerol. The discovery of this pathway resulted from our finding that WAT contains both pyruvate carboxylase and the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK-C) (10), two enzymes thought at the time to be involved only in gluconeogenesis. In addition, glyceroneogenesis was predicted to be important in lipid metabolism in ruminants (13), because these animals do not derive glucose from the digestion of dietary carbohydrate, because of the activity of rumen microflora. Rather, they rely on the synthesis of glucose by hepatic and renal gluconeogenesis; thus glucose is at a premium in all ruminants, especially during pregnancy and lactation (14). On this basis, we sought an alternative pathway for the generation of the 3-glycerol phosphate that is required for triglyceride synthesis during fasting in ruminant animals. In fact, in all mammals glucose is a critical fuel for the metabolism of a number of tissues, such as the brain and red blood cells. Thus the importance of an alternative source of 3-glycerol phosphate during fasting, other than glucose, is evident.

In the initial studies that established the existence of the pathway of glyceroneogenesis, we demonstrated that the addition of pyruvate to rat epididymal adipose tissue, incubated *in vitro*, reduced FFA release by ~65%, while not altering lipolysis (as determined by the amount of glycerol released into the medium) because of increased FFA re-esterification (11, 15, 16). These findings suggested a potential physiological role for glyceroneogenesis in the re-esterification of FFA in WAT during fasting, thereby controlling both the release of FFA and subsequently ketogenesis (11). Despite these early studies, the pathway has remained largely ignored (17) or cited only sparingly in the literature over the past 35 years. A recent perusal of the archives of PubMed indicated that only 28 published papers referred to glyceroneogenesis in their titles; the subject has yet to be introduced into text books of general biochemistry.

This situation has slowly changed over the past several years (18) due in part to the availability of genetically modified mice in which the gene for PEPCK-C has been either specifically deleted (19, 20) or overexpressed in adipose tissue (21). Modifying the expression of the gene for PEPCK-C is critical because this enzyme,

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[1] The abbreviations used are: FFA, free fatty acid(s); WAT, white adipose tissue; BAT, brown adipose tissue; PPAR, peroxisome proliferator-activated receptor; PPARE, PPAR response element; PEPCK, phosphoenolpyruvate carboxykinase; C/EBP, CCAAT/enhancer-binding protein; CREB, cAMP-response element-binding protein.

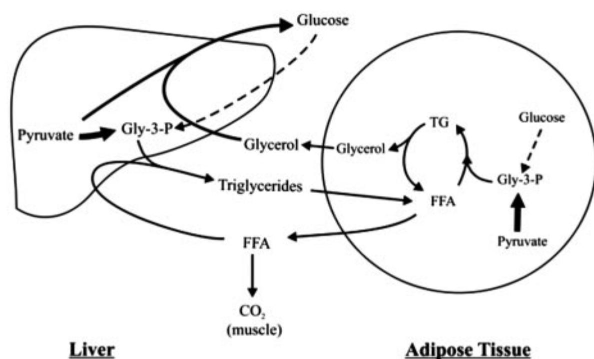

FIG. 1. **The triglyceride/fatty acid cycle in mammals.** FFA released by WAT is re-esterified back to triglyceride (*TG*) in that tissue or in the liver as part of a general cycle that accounts for about 60% of the FFA released by lipolysis of triglyceride in WAT. Table I presents a systematic quantitation of the level of recycling of FFA in the adipose tissue and the liver.

TABLE I
Measurement of the triglyceride/fatty acid cycle in vivo

	FFA Ra[a]	Recycled		
		Adipose tissue	Systemic	Total
	$\mu mol \cdot kg^{-1} \cdot min^{-1}$	%	%	%
Adults				
Fast (12 h)	7.2	8.3	40.3	48
Burn	24.6	41.1	23.2	64
Newborn infants				
Healthy	28.5			76
Malnourished	36.6			78
Effect of leptin (rabbits)				
Basal	29.1	28	56	85
Leptin	44.4	34	49	84

[a] FFA Ra is the rate of appearance of FFA from adipose tissue. The amount of FFA recycled is calculated as described in detail in the specific references listed. Data from the following publications were used to construct this table: Ref. 8, fasting adults; Ref. 4, severe burn patients; Ref. 9, newborn humans; Ref. 6, effect of leptin on fatty acid recycling.

which catalyzes the synthesis of phosphoenolpyruvate from oxalacetate, is generally considered to be the pace-setting step in both gluconeogenesis and glyceroneogenesis. PEPCK-C is encoded by a single copy gene, which is expressed to the greatest extent in the liver and kidney cortex and both WAT and brown adipose tissue (BAT). Both the activity of PEPCK-C and the number of enzyme molecules is acutely regulated by alterations in the rate of transcription of the PEPCK-C gene (22), in response to various dietary, hormonal, and environmental stimuli.

The tissue-specific expression of the gene for PEPCK-C is due to regulatory elements in the gene promoter that bind tissue-specific transcription activators to direct its expression (22). Because deletion of the gene for PEPCK-C is neonatal lethal (19), we have generated a targeted mutation in embryonic stem cells of the PPARγ2 binding site in the promoter of the PEPCK-C gene in mice to assess the role of this enzyme in adipose tissue *in vivo* (20). The PPARγ2 binding site is required for the adipose tissue-specific expression of the gene (23). The mutation abolished PEPCK-C gene expression only in WAT and to a smaller extent (50%) in BAT of the homozygous descendent mice (PEPCK-PPARE$^{-/-}$ mice). The mutation virtually abolished glyceroneogenesis in WAT of PEPCK-PPARE$^{-/-}$ mice, as determined by their inability to synthesize glyceride-glycerol from pyruvate; this establishes PEPCK-C as a key enzyme in glyceroneogenesis. The PEPCK-PPARE$^{-/-}$ mice also lost considerable triglyceride from their adipose tissue, and about 30% of the animals became lipodystrophic as adults (20). The lipodystrophy noted in the PEPCK-PPARE$^{-/-}$ mice differs considerably from that found in other models of this disorder (24) because the animals do not have altered glucose metabolism, except for a very moderate hyperglycemia noted in older mice.

Further support for the importance of glyceroneogenesis in adipose tissue was provided by the experiments of Franckhauser *et al.* (21) who overexpressed a chimeric transgene containing the PEPCK-C structural gene linked to the aP2 promoter in transgenic mice. The gene was expressed at high levels, specifically in WAT because of the specificity of the aP2 gene promoter. Adult transgenic mice had greatly enhanced rates of glyceroneogenesis and higher levels of triglyceride synthesis in their WAT; the animals were also markedly obese and did not exhibit signs of altered glucose metabolism. These findings, together with those of Olswang *et al.* (20), provide strong support for the pathway of glyceroneogenesis in WAT and the key role that is played by PEPCK-C in controlling the turnover of triglyceride during fasting.

More recently an analysis of 16,757 genes in *Caenorhabditis elegans* using the RNA-mediated interference technique indicated that disruption of the gene for PEPCK results in a reduced or disorganized pattern of fat deposition in the worm (25).

The importance of glyceroneogenesis in controlling triglyceride turnover in WAT is supported by other lines of evidence. Glucose is the major precursor of 3-glycerol phosphate for triglyceride synthesis in this tissue in the fed state. During diabetes, there is both an elevated level of lipolysis and a greatly diminished rate of transport of glucose into the adipocyte, resulting in mobilization of triglyceride from adipose tissue. Adipocyte-specific deletion of the gene for the transporter that is required for glucose entry into the adipocyte (GLUT4) generated mice that were insulin-resistant. Despite this, these mice did not have a loss of triglycerides from WAT (26). Thus, there must be an alternative source of 3-glycerol phosphate for triglyceride synthesis in WAT in the GLUT4-deficient mice. It is likely that these mice will have an enhanced activity of glyceroneogenesis in their WAT in order to maintain triglyceride homeostasis.

Finally, it is of interest that the gene for the mitochondrial dicarboxylate transporter is highly expressed in WAT and the level of its mRNA is induced by fatty acids and inhibited by insulin (27). The dicarboxylate transporter is required for the movement of malate in exchange for other anions, such as α-ketoglutarate, from the mitochondria to the cytosol. This pathway would provide the major route for the generation of cytosolic oxalacetate, a substrate of PEPCK-C in glyceroneogenesis.

Glyceroneogenesis in Brown Adipose Tissue

It has been known for many years that BAT contains considerable activity of PEPCK-C (about 10 times that of WAT) (28) yet the physiological function of the enzyme in that tissue has not been formally established. The activity of PEPCK-C can be induced in BAT by corticosteroids (29), by the administration of norepinephrine (30) and thyroid hormone (31), by a diet high in protein and devoid of carbohydrate (30) and can be inhibited by insulin (31). Brito *et al.* (32), using isotopic tracers, have shown that feeding rats a high protein, carbohydrate-free diet will markedly induce the level of glyceroneogenesis from alanine, pyruvate, and lactate in BAT and increase the activity of PEPCK-C 4-fold, leading to increased re-esterification of FFA. This pathway may play a critical role in determining the rate of delivery of fatty acids to the mitochondria for energy generation required for nonshivering thermogenesis. In support of this concept, a high protein, carbohydrate-free diet also reduces the thermogenic capacity of BAT in rats (33). Taken together, these findings suggest that glyceroneogenesis plays a critical role in thermogenesis in BAT by controlling the rate of formation of 3-glycerol phosphate required for triglyceride synthesis in that tissue. There is one surprising aspect of this story; unlike WAT, BAT has considerable activity of glycerol kinase that can form the 3-glycerol phosphate for triglyceride synthesis directly from glycerol. However, most of the preformed fatty acids are re-esterified to triglyceride using 3-glycerol phosphate generated via glyceroneogenesis (32). It is also likely that in these mice fed a carbohydrate-free diet, glyceroneogenesis plays a key role in providing the 3-glycerol phosphate required to ensure triglyceride synthesis from dietary fatty acids in BAT during the fed state. Glyceroneogenesis is, of course, also critical in controlling the rate of triglyceride re-esterification after norepinephrine stimulation due to cold exposure. In this regard, Feldman and Hirst (29) reported that exposing rats to the cold caused a marked decrease in PEPCK-C in BAT. This would mean a decrease in the rate of FFA re-esterification and an increase in the delivery of fatty acid to the mitochondria to maintain thermogenesis.

In a review published in 1975, Hahn and Novak (28) point out that although BAT has 10 times the activity of PEPCK-C as compared with WAT (based on cellular protein content) the rate of glyceroneogenesis, as measured by the rate of incorporation of labeled pyruvate into triglyceride, is 4 times greater in WAT than in BAT. They proposed that the "extra" PEPCK-C activity is involved in a futile cycle in which the enzyme uses the GTP generated in the citric acid cycle by succinyl-CoA synthase to form phosphoenolpyruvate from oxalacetate, which is then converted to pyruvate via pyruvate kinase. Further, the pyruvate is carboxylated to oxalacetate or decarboxylated to acetyl-CoA to replenish the citric acid cycle. The forward progress of the citric acid cycle will generate malate that leaves the mitochondria to maintain the supply of cytosolic oxalacetate for PEPCK-C. The net result is a futile cycle in which one molecule of GTP is used by PEPCK-C and one molecule of ATP by pyruvate carboxylase, with only one molecule of ATP being generated by pyruvate kinase. This proposed futile cycle could aid in the generation of heat by the BAT. The ablation of expression of the gene for PEPCK-C in BAT should result in a lower rate of futile cycling of pyruvate as well as a decrease of triglyceride fatty acid cycling leading to a decrease of triglyceride in the tissue. This was confirmed in the mouse model in which the PPARγ binding site in the PEPCK-C gene promoter was mutated, thereby ablating expression of the gene in WAT and BAT (but in no other tissue); the mice had a marked loss of triglyceride from both tissues (20). Thus, glyceroneogenesis in BAT is quantitatively important for maintaining the appropriate level of triglycerides in the cell. Because fatty acid re-esterification is itself a futile cycle (6 molecules of ATP are required to activate 3 molecules of fatty acid to 3 acyl-CoAs for triglyceride synthesis), active rates of triglyceride synthesis in BAT could add to the heat production generated by fatty acid-induced uncoupling via UCP-1.

Can Glycerol Be Used Directly for the Synthesis of Glyceride-Glycerol?

A central premise of WAT metabolism is that the glycerol released during lipolysis *cannot* be phosphorylated and used for triglyceride synthesis because this tissue has a negligible activity of glycerol kinase. Most of the glycerol released during fasting is taken up by the liver and converted to glucose, although some glycerol can be used by BAT (28) and muscle (34, 35). Because some FFA is re-esterified back to triglyceride by WAT during fasting, the rate of glycerol release by that tissue is routinely used as a measure of lipolysis. Over the years there have been several reports of glycerol kinase activity in WAT, but the reported activity is not great enough to support the high rates of FFA re-esterification that would be required for triglyceride synthesis when glucose is limiting, such as occurs in this tissue during fasting. Recently, Guan *et al.* (17) proposed that WAT contains sufficient glycerol kinase to play a role in triglyceride homeostasis in this tissue in the presence of thiazolidinediones. As predicted, they detected very low levels of glycerol kinase in control adipocytes but upon the addition of concentrations of 0.1 μM (or higher) of rosiglitazone, they noted the presence of glycerol kinase activity. In addition, the injection of ciglitazone (100 mg/kg of body weight/day for 4 days) to Ob/Ob mice caused an induction of glycerol kinase in the WAT of the animals. Interestingly, non-obese mice had far less glycerol kinase induction after ciglitazone injection, and control animals had negligible levels of glycerol kinase in their adipose tissue. Adipocytes responded to the addition of rosiglitazone by greatly increasing the incorporation of isotopically labeled glycerol into glyceride-glycerol and by reducing FFA release (after 48 h of rosiglitazone treatment). We can conclude from this study that the thiazolidinediones can induce the expression of the gene for glycerol kinase in WAT and can thus stimulate FFA re-esterification in that tissue. However, the levels of glycerol kinase in WAT are negligible in basal, unstimulated conditions.

Thiazolidinediones are ligands for PPARγ, a member of the steroid hormone/thyroid hormone/retinoid receptor superfamily of transcription factors. PPARγ causes the marked and rapid induction of PEPCK-C gene expression in WAT (36) and has been shown to bind to specific sites in the PEPCK-C gene promoter (36–38). In addition, PPARγ is required for the tissue-specific expression of the gene for PEPCK-C in WAT (23). Thus the administration of thiazolidinediones would stimulate transcription of the gene for PEPCK-C (39), as well as the gene for glycerol kinase, suggesting that the increase in FFA re-esterification noted by Guan *et al.* (17) could be due as well to the thiazolidinedione induction of PEPCK-C gene transcription (18, 39), which in turn would be accompanied by an increase in glyceroneogenesis.

The relative importance of glyceroneogenesis *versus* glycerol kinase in the thiazolidinedione-induced decrease in the release of fatty acid from WAT has been evaluated in a recent study by Tordjman *et al.* (40). They noted that rosiglitazone induced glycerol kinase activity in isolated adipocytes from a very low basal activity of 0.2 nmol/min/mg of protein to 0.5 nmol/min/mg of protein after 72 h. In contrast, the activity of PEPCK-C in adipose tissue was 5 nmol/min/mg of protein in the basal state and 14 nmol/min/mg of protein after the addition of rosiglitazone. The overall rate of fatty acid re-esterification resulting from glyceroneogenesis, as determined in this study, was 3–5 times higher than the rates for glycerol conversion to glyceride-glycerol in adipocytes incubated with rosiglitazone. These authors conclude that the major effect of rosiglitazone in decreasing the levels of plasma FFA is due to a stimulation of PEPCK-C gene transcription leading to an increase in fatty acid re-esterification via glyceroneogenesis and that the induction of glycerol kinase is a relatively minor component of the response of the WAT to thiazolidinediones (40).

Glyceroneogenesis Occurs in the Liver

Although glyceroneogenesis was first described in WAT, its function in the liver was not recognized until the work of Botion *et al.* (41), who reported high rates of glyceroneogenesis in the livers of rats fed a high protein, carbohydrate-free diet. The presence of glyceroneogenesis in the liver was unexpected for two reasons. First, it is not intuitive that the liver would make triglyceride during periods of fasting, when it is actively involved in gluconeogenesis and is using fatty acids from adipose tissue as a source of energy to support this process (and urea synthesis). Second, the liver can readily use the considerable glycerol released from WAT during lipolysis as a source of 3-glycerol phosphate (as mentioned above, the liver has considerable glycerol kinase activity (42)). Glycerol kinase readily phosphorylates glycerol, which should make hepatic glyceroneogenesis redundant. However, experiments by Kalhan *et al.* (43), using stable isotopes *in vivo*, demonstrated that glycerol contributed only about 3% of the glyceride-glycerol noted in the triglyceride in the blood of human subjects after an overnight fast. These studies have been extended to include more human subjects under a variety of experimental conditions, such as prolonged fasting and diabetes, and support our original observations.[2] Plasma glycerol thus contributes only marginally to the synthesis of glyceride-glycerol of hepatic triglyceride present in plasma very low density lipoprotein, whereas glyceroneogenesis provides about 65% of the glyceride-glycerol. The underlying biochemical mechanisms that regulate the flow of carbon to glyceride-glycerol in the liver during starvation remains to be elucidated.

Regulation of Glyceroneogenesis in Liver and WAT

The data outlined above underline the importance of the coordinate regulation of PEPCK-C gene transcription in the liver and WAT. Active glyceroneogenesis in both liver and WAT requires coordination of triglyceride turnover in the two tissues because PEPCK-C activity catalyzes the rate-limiting step in triglyceride re-esterification in both tissues (glyceroneogenesis). Lipid is released from WAT as FFA and from the liver as triglycerides. *Thus, glyceroneogenesis affects lipid metabolism in opposite ways in the two tissues; it restrains FFA release from WAT (11) and enhances it (in the form of triglyceride) from the liver (44).* It has been shown in rats that adrenalectomy enhances glyceroneogenesis and diminishes FFA release from adipose tissue *in vitro* (15, 45). The addition of dexamethasone to cultured hepatocytes increased the synthesis of triglycerides and apolipoproteins E and B and stimulated the release of very low density lipoproteins to the medium (44). How then is lipid homeostasis coordinated between the two tissues? We propose that the reciprocal regulation of PEPCK-C gene transcription by glucocorticoids provides a mechanism for such coordination

[2] S. C. Kalhan, unpublished results.

because it represses PEPCK-C gene transcription in WAT, while enhancing it in the liver.

Several hormones work together to regulate PEPCK-C activity and both gluconeogenesis and glyceroneogenesis (15, 46, 47) and do so in a differential manner in specific tissues that express the enzyme. The best example is the regulation by glucocorticoids that induce PEPCK-C gene transcription in the kidney and liver (48) but repress it in WAT (49). Moreover, the glucocorticoid-mediated repression of PEPCK-C synthesis is effective even under basal concentrations of circulating glucocorticoids, because removal of the adrenals enhances enzyme synthesis by 3-fold (50).

The molecular mechanisms that underlie the reciprocal regulation of PEPCK-C gene transcription by glucocorticoids are not well understood. This differential regulation of PEPCK-C gene transcription most likely involves the interaction of tissue-specific transcriptional activators that results in an induction of PEPCK-C gene transcription (liver and kidney) or repression (WAT). We have recently found that members of the C/EBP family of transcription factors are involved in the repression of PEPCK-C gene transcription in WAT (51). DNase I footprint analysis of the rat PEPCK-C gene promoter using nuclear proteins from adipocytes that had been treated with dexamethasone indicated that the hormone treatment interfered with the binding of nuclear proteins to the C/EBP recognition sites in the promoter. Furthermore, PPARγ2 and both C/EBPα and C/EBPβ activated transcription from the PEPCK-C gene promoter in NIH3T3 fibroblasts. However, dexamethasone (in the presence of the glucocorticoid receptor) inhibited the activation of transcription by members of the C/EBP family but had no effect on PPAR-stimulated transcription (51). This repression of transcription of PEPCK-C gene transcription by glucocorticoids does not require DNA binding of the receptor, suggesting a mechanism of repression that involves a co-regulatory protein such as CREB-binding protein/p300. Interestingly, mice that are homozygous for a deletion in the gene for C/EBPβ have 4 times the concentration of PEPCK-C mRNA and twice the activity of the enzyme in WAT as compared with control littermates.[3] These findings suggest that C/EBPβ is important for the negative control of PEPCK-C gene transcription in WAT. The relative importance of the various transcription factors that control PEPCK-C gene transcription in the liver and WAT remains to be determined. Despite the progress made in the elucidation of the reciprocal hormonal control of PEPCK-C gene transcription in various tissues, its physiological meaning remained an enigma until recent metabolic studies indicated the presence of active hepatic glyceroneogenesis (41, 43).

A Summary Remark

The ability to genetically manipulate animals has made a major impact on our understanding of metabolic processes. The pathway of glyceroneogenesis was first described more than 35 years ago with little subsequent interest from the scientific community, despite its potential role in controlling fatty acid recycling in both WAT and liver. Glyceroneogenesis also provides a mechanistic explanation for the findings of Bernard Houssay (52) and of Long and Leukins (53) that glucocorticoids are required for the development of diabetes in animals. Without glucocorticoids the liver will not make as much glucose (due to a lower activity of PEPCK-C) and will not re-esterify fatty acids to triglyceride as readily. WAT will also have higher rates of glyceroneogenesis, resulting in less FFA release. A higher concentration of both glucose and FFA in the blood is a characteristic of diabetes. Thus, glucocorticoids with their coordinating role in controlling expression of the gene for PEPCK-C, both WAT and liver, would be required for the development of diabetes mellitus. Viewed in this way, the physiological significance of PEPCK-C and glyceroneogenesis in WAT and liver can be better understood.

[3] C. M. Croniger and R. W. Hanson, unpublished results.

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