Impaired amino acid metabolism contributes to fasting-induced hypoglycemia in fatty acid oxidation defects

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Received June 21, 2013; Revised July 10, 2013; Accepted August 3, 2013

The importance of mitochondrial fatty acid β-oxidation (FAO) as a glucose-sparing process is illustrated by patients with inherited defects in FAO, who may present with life-threatening fasting-induced hypoketotic hypoglycemia. It is unknown why peripheral glucose demand outpaces hepatic gluconeogenesis in these patients. In this study, we have systematically addressed the fasting response in long-chain acyl-CoA dehydrogenase-deficient (LCAD KO) mice. We demonstrate that the fasting-induced hypoglycemia in LCAD KO mice was initiated by an increased glucose requirement in peripheral tissues, leading to rapid hepatic glycogen depletion. Gluconeogenesis did not compensate for the increased glucose demand, which was not due to insufficient hepatic glucogenic capacity but rather caused by a shortage in the supply of glucogenic precursors. This shortage in supply was explained by a suppressed glucose—alanine cycle, decreased branched-chain amino acid metabolism and ultimately impaired protein mobilization. We conclude that during fasting, FAO not only serves to spare glucose but is also indispensable for amino acid metabolism, which is essential for the maintenance of adequate glucose production.

INTRODUCTION

Glucose and fatty acids compete as sources of energy in a process that is known as the glucose-fatty acid or Randle cycle (1). In the fasted state, mitochondrial fatty acid β-oxidation (FAO) is stimulated by an increased availability of fatty acids caused by enhanced lipolysis in adipose tissue, which is mediated by changes in the insulin/glucagon ratio. Enhancing hepatic FAO is crucial for the production of ketone bodies that can serve as an alternative energy source. This fasting response is thought to preserve glucose, which is an indispensable energy source for the brain. During fasting, glucose is produced by the liver through glycogenolysis (glycogen breakdown) and gluconeogenesis (de novo synthesis). For the latter process, specific glucogenic amino acids, lactate and glycerol are used. The generation of glucogenic amino acids by tissue proteolysis is largely responsible for the net addition of carbons into the glucose pool via anaplerosis, but this process has to be tightly controlled in order to prevent excessive erosion of protein mass (2,3). Taken together, fatty acids are generally considered an important yet nonessential alternative for glucose in the maintenance of energy homeostasis.

Patients with an inherited defect in one of the enzymes involved in FAO have a low fasting tolerance. During periods

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of fasting and other metabolically demanding conditions such as febrile illness, these patients may present with hypoketotic hypoglycemia, which may progress into life-threatening ‘Reye-like syndrome’ (4). The clinical presentation of these patients illustrates that FAO is indispensable during fasting. Yet, the pathogenetic mechanisms underlying hypoglycemia have only partially been elucidated. Based on the concept of the Randle cycle, patients with an FAO disorder are expected to have an increased glucose demand because they have a diminished capacity to generate energy from fatty acids and ketones. Indeed, patients with carnitine palmityltransferase 2 deficiency or very long chain acyl-CoA dehydrogenase (VLCAD) deficiency compensate impaired FAO during prolonged low-intensity exercise by increased glucose metabolism—in this case, driven by enhanced muscle glycogenolysis (5,6).

An increased peripheral glucose demand can progress to hypoglycemia when gluconeogenesis is not sufficiently enhanced in order to compensate for the increased glucose uptake. It has been proposed that gluconeogenesis is inhibited in patients with an FAO defect, but the relationship between gluconeogenesis and FAO is complex. Delivery of free fatty acids is able to stimulate gluconeogenesis in vitro (7) and in vivo (8). In addition, pharmacological inhibition of FAO suppresses gluconeogenesis in perfused rat liver (9), which is caused by low levels of acetyl-CoA that limit the activity of the rate-controlling enzyme pyruvate carboxylase (10). Alternatively, gluconeogenesis could be impaired due to a shortage of energy or glucogenic precursors.

To elucidate the pathogenetic mechanisms leading to hypoglycemia in FAO disorders, we have systematically addressed the major metabolic adaptations to fasting in the long-chain acyl-CoA dehydrogenase (LCAD) KO mouse. The LCAD KO mouse represents a model for human FAO disorders that resembles human VLCAD deficiency in several aspects (11–16). We show that fasting-induced hypoglycemia in these mice is indeed explained by a failure to appropriately enhance hepatic gluconeogenesis in order to compensate for an increased peripheral glucose demand. Unexpectedly, we provide evidence that this inability is caused by a limiting availability of glucogenic precursors.

RESULTS

Rapid onset of hypoglycemia during fasting in LCAD KO mice

We first evaluated key markers for FAO defects in overnight-fasted WT and LCAD KO mice. When compared with WT mice, fasted LCAD KO mice displayed hypoketotic hypoglycemia in combination with elevated free fatty acid and C14:1-carnitine levels. These plasma metabolite changes upon fasting strongly resemble human VLCAD deficiency (Fig. 1A, Supplementary Material, Table S1). Remarkably, the LCAD KO mice were already hypoglycemic during the postabsorptive phase preceding the development of hypoketosis (Supplementary Material, Table S1). Indeed, a short period of fasting at the initiation of the dark phase (Supplementary Material, Fig. S1A), but also during the light phase (Fig. 1B), caused a rapid decline in blood glucose in LCAD KO mice, whereas WT mice kept their blood glucose levels relatively stable. In contrast, ad libitum-fed LCAD KO mice had similar blood glucose levels as WT mice and did not develop hypoglycemia during the light phase (Fig. 1B). The hypoglycemia was not due to hyperinsulinism (Supplementary Material, Fig. S1B) and rapidly normalized upon refeeding (Supplementary Material, Fig. S1C). Thus, LCAD KO mice become hypoglycemic soon after the initiation of fasting.

VLCAD and LCAD have previously been shown to have a partially overlapping function in murine FAO (12,13,15). To exclude the possibility that induced activity of VLCAD was compensating for deficient LCAD activity, we measured the acyl-CoA dehydrogenase enzyme activities. In the postabsorptive and overnight-fasted state, acyl-CoA dehydrogenase activities in the liver, gastrocnemius muscle and heart of LCAD KO mice were similarly reduced when compared with WT mice (Supplementary Material, Fig. S2). In addition, hepatic VLCAD (Acadvl) expression levels were similar between WT and LCAD KO animals in the postabsorptive and overnight-fasted state (Supplementary Material, Fig. S2). Thus, the enzymatic defect in FAO in LCAD KO mice is not compensated for upon overnight fasting.

Efficient glucose use despite hypoglycemia in fasted LCAD KO mice

To define the cause of hypoglycemia in fasted LCAD KO mice, we assessed glucose metabolism using stable isotope
methodology in non-anesthetized mice (Supplementary Material, Fig. S3A). During the infusion experiment, blood glucose levels were stable in both groups of mice and LCAD KO mice remained hypoglycemic (Supplementary Material, Fig. S3B).

We found that the metabolic clearance rate (MCR) of glucose was increased 1.5-fold (Fig. 2A), whereas the endogenous glucose production (EGP) was slightly decreased in LCAD KO animals (Fig. 2B). To quantify the glucose uptake into organs,

Figure 2. Glucose metabolism in fasted LCAD KO mouse. (A) MCR and (B) EGP. (C) Glucose uptake in soleus muscle ($n = 5$ for WT and $n = 6$ for LCAD KO). (D) Hepatic glycogen and (E) G6P levels. (F) Hepatic fluxes through the enzymes of hepatic glucose metabolism during the last 3 h of the infusion experiment are displayed. Glucose balance represents G6Pase flux—hexokinase flux. Glycogen balance represents glycogen synthase flux—glycogen phosphorylase flux. For all metabolic flux and metabolite measurements, $n = 8$ per group. Error bars indicate SEM.
we injected labeled deoxyglucose in overnight-fasted WT and LCAD KO mice and measured its conversion to phosphodeoxyglucose in several organs. Soleus muscle (Fig. 2C, 3-fold) and heart (Supplementary Material, Fig. 3C, 18-fold) of LCAD KO mice displayed a markedly higher glucose uptake, whereas glucose uptake into brown and white adipose tissue, gastrocnemius muscle and brain was similar between genotypes (Supplementary Material, Fig. 3C). These results show that, although having lower levels of circulating glucose, LCAD KO mice maintain a highly efficient peripheral glucose uptake.

Glucogenesis in fasted LCAD KO mice does not compensate for hypoglycemia

Next we assessed the origins of hepatic glucose production, using mass isotopomer distribution analysis in fasted WT and LCAD KO mice. The net glucose output, defined as the glucose balance, was similar in WT and LCAD KO mice. This value combines glucose production from glucogenogenesis and glycogenolysis and is calculated as the difference between glucose-6-phosphatase (G6Pase) and glucokinase fluxes. We observed that the G6Pase flux in LCAD KO mice was lower than in WT mice due to a reduced EGP and a reduced glucose cycling (Fig. 2F). Glucokinase flux also was reduced in LCAD KO mice, most likely due to hypoglycemia, which caused reduced glucose uptake and cycling. This parallel reduction in the flux of G6Pase and glucokinase resulted in the equal hepatic glucose output in both groups of mice.

Fasted LCAD KO mice had depleted hepatic glycogen stores, resulting in a low glycogen phosphorylase flux (Fig. 2D and F). However, because the glycogen synthase flux was also lower in the LCAD KO mouse, the glycogen balance was almost identical in both groups (Fig. 2F). To further substantiate hepatic glycogen deficiency, we tested the response of overnight-fasted WT and LCAD KO mice to a glucagon challenge. Whereas WT mice displayed a marked increase in blood glucose levels upon glucagon injection, indicating mobilization of hepatic glycogen stores, this hyperglycemic response was completely absent in LCAD KO mice, which is consistent with depleted hepatic glycogen stores (Supplementary Material, Fig. S3D).

Remarkably, glucogenogenesis flux was also similar in LCAD KO and WT mice (Fig. 2F). Combined, these changes in glucose fluxes resulted in lower hepatic glucose-6-phosphate (G6P) levels in the LCAD KO mouse (Fig. 2E). Thus, de novo synthesis of G6P and, as a consequence, the net glucose output of the liver is not enhanced to compensate for the prevailing hypoglycemia in fasting LCAD KO mice.

Changes in hepatic gene expression reflect altered glucose metabolism in fasted LCAD KO mice

To complement the biochemical characterizations with molecular expression profiles, we determined hepatic mRNA levels of genes involved in glucose metabolism in overnight-fasted WT and LCAD KO mice (Table 1). Expression of pyruvate dehydrogenase kinase 4 (Pdk4), which can limit pyruvate oxidation, was induced 10-fold in LCAD KO mice. Expression of Glut1 (Slc2a1), glycogen synthase (Gys2, liver isoform), G6Pase (G6pc), phosphoenolpyruvate carboxykinase (Pck1) and pyruvate carboxylase (Pcx) did not differ between WT and LCAD KO mice. In contrast, expression of Glut2 (Slc2a2), glucokinase (Gck), glycogen phosphorylase (Pyg2), fructose-1,6-bisphosphatase (Fbp1) and pyruvate kinase (Pklr) were significantly decreased in fasted LCAD KO mice. Glut2 and glucokinase both have low affinities for glucose and serve as a hepatic glucose sensor. Therefore, their decreased expression in combination with hypoglycemia likely contributes to the observed reduced hepatic glucose uptake and cycling. Thus, changes in hepatic gene expression reflect and may contribute to the altered glucose metabolism in fasted LCAD KO mice.

Targeted metabolomics reveal that hypoketotic hypoglycemia in fasted LCAD KO mice coincides with changes in amino acid metabolism

To identify the biochemical mechanisms underlying the failure to compensate for fasting-induced hypoglycemia by enhancing glucogenogenesis, we performed targeted quantitative metabolomics on blood and plasma of LCAD KO and WT mice in the postabsorptive and in the overnight-fasted state (Supplementary Material, Table S1). We used the targeted metabolomics data set for two-dimensional hierarchical clustering. In one dimension, this analysis perfectly clustered the four different groups of mice according to genotype and duration of fasting (Fig. 3A). Importantly, clustering in the other dimension

| Table 1. Hepatic gene expression pattern in overnight-fasted WT and LCAD KO mice |
|-----------------|------------------|-----------------|
| Gene            | Protein          | WT              | LCAD KO         | Sig.  |
| Gck             | Glucokinase      | 1.00 ± 0.24     | 0.31 ± 0.28     | ***  |
| Slc2a2          | Glut2            | 1.00 ± 0.24     | 0.32 ± 0.07     | ***  |
| Pckr            | Pyruvate kinase  | 1.00 ± 0.28     | 0.51 ± 0.16     | **   |
| Pyg2            | Liver glycogen   | 1.00 ± 0.19     | 0.58 ± 0.13     | **   |
| Fbp1            | Fructose-1,6-bisphosphatase | 1.00 ± 0.30 | 0.66 ± 0.10 | * |
| Pklr            | Pck              | 1.00 ± 0.22     | 0.79 ± 0.10     | 0.08  |
| Gys2            | Glycogen synthase| 1.00 ± 0.24     | 1.08 ± 0.11     | #    |
| Slc2a1          | Glut1            | 1.00 ± 0.35     | 1.15 ± 0.16     | #    |
| Pcx             | Pyruvate carboxylase | 1.00 ± 0.24 | 1.19 ± 0.18 | #  |
| G6pc            | Glucose-6-phosphatase | 1.00 ± 0.46 | 1.20 ± 0.44 | #  |
| Pdk4            | Pyruvate dehydrogenase kinase 4 | 1.00 ± 1.06 | 9.84 ± 2.75 | *** |

Values are the average ± SD. Each group consists of five animals. Significance (Sig.) is indicated as follows: # = not significant, * < 0.05, ** < 0.01 and *** < 0.001.
revealed specific clusters of metabolites, such as the long-chain acylcarnitines (Fig. 3A). These metabolites are known to accumulate in plasma with a profile reflecting the substrate specificity of murine LCAD (12,15,17).

Interestingly, a second identified metabolite cluster was related to amino acid and pyruvate metabolism and contained lactate, pyruvate, lysine, glycine, glutamine, alanine and serine, as well as propionyl-, C5- and C5OH-carnitine, which are derived from intermediates in branched-chain amino acid (BCAA) degradation (Fig. 3A). The levels of all these metabolites were low in overnight-fasted LCAD KO mice when compared with WT mice. Similar to glucose (Fig. 1B), plasma alanine levels were already significantly lower in postabsorptive LCAD KO mice (Supplementary Material, Table S1). In addition, there was a correlation between plasma alanine and blood pyruvate levels within individual mice of the fasted LCAD KO group (Fig. 3B). This correlation illustrates the fact that tissue pyruvate levels drive alanine formation via the alanine aminotransferase (ALAT) reaction, which is a crucial step in the glucose–alanine cycle that serves to transport nitrogen derived from peripheral protein degradation to the liver for subsequent conversion into urea. Synthesis of glutamine is the
second major means of nitrogen removal from the periphery. During fasting, muscle glutamine synthetase catalyzes the ATP-dependent conversion of glutamate and ammonia into glutamine (18). Thus, fasted LCAD KO mice have lower levels of glucogenic precursors, including alanine and glutamine, indicating that this phenomenon may be related to reduced protein degradation. This was further evidenced by the significantly lower levels of lysine, a marker for muscle protein degradation (19). The levels of BCAAs and their relative contribution to the total free amino acid pool were significantly higher in fasted LCAD KO mice when compared with WT mice (Figs 3A and 4E, Supplementary Material, Table S1). Combined with the decreased propionyl-, C5- and C5OH-carnitine levels, this indicates that BCAA oxidation was reduced in fasted LCAD KO mice.

Since clustering techniques only reveal groups of important metabolites, we extended our analysis with supervised classification using Random Forest (RF), which assigns importance to individual metabolites. In our analysis, we included selected ratios between metabolites considered as substrate-product pairs. These ratios can serve as a proxy for the conversion rate of these metabolites. For postabsorptive as well as overnight-fasted mice, RF accurately separated the mice by genotype and ranked the top 10 parameters contributing to the separation of WT and LCAD KO mice by their ‘mean decrease accuracy’. Whereas postabsorptive mice were primarily classified according to several long-chain acylcarnitines and their ratios, the separation of overnight-fasted mice was also driven by propionylcarnitine and the ratio between the BCAAs and alanine, thus highlighting the changes in amino acid metabolism (Fig. 3C).

A disturbed protein homeostasis limits availability of glucogenic precursors in fasted LCAD KO mice

Our metabolomics data indicate that gluconeogenesis could not be enhanced in fasted LCAD KO mice due to limiting availability of glucogenic precursors. We, therefore, tested whether gluconeogenesis could be rescued by a bolus injection of L-alanine, a major glucogenic precursor. L-alanine equally raised plasma levels of lysine, a marker for muscle protein degradation (19). The mechanistic target of rapamycin complex (mTORC)/S6-kinase pathway integrates amino acid availability with insulin levels and as such controls protein homeostasis as well as many other biosynthetic processes. Therefore, we studied this signaling pathway in WT and LCAD KO mice in the postabsorptive as well as the fasted state. The insulin targets Akt, GSK-3β, p70S6-kinase and MAPK were all hypophosphorylated in the liver of overnight-fast WT and LCAD KO mice, consistent with low insulin levels (Supplementary Material, Fig. S4B). Interestingly, the ribosomal protein S6 (rpS6) remained hyperphosphorylated after overnight fasting in LCAD KO mice, whereas it was hypophosphorylated in WT mice (Fig. 4C). Also in hearts of fasted LCAD KO mice, the ratio of phosphorylated rpS6 over total rpS6 was 3-fold higher than in WT hearts (Fig. 4D). These data suggest that the disturbed protein degradation in liver and periphery of fasted LCAD KO mice may be due to increased mTORC signaling.

Leucine, glutamine and arginine have been suggested as regulators of mTORC activity (21). To identify potential contributors to the sustained mTORC activation, we measured amino acid levels in the liver, skeletal muscle and heart of overnight-fast WT and LCAD KO mice (Supplementary Material, Tables S2 - S4) and compared these with plasma amino acid levels (Supplementary Material, Table S1). Arginine levels were not different, but glutamine levels were lower in muscle and heart of fasted LCAD KO mice. In addition, the contribution of BCAAs to the total free amino acid pool was larger in the liver, muscle and heart of LCAD KO mice compared with WT controls (Fig. 4E). These findings further substantiate the changes in amino acid metabolism of fasted LCAD KO mice and may provide an explanation for the sustained mTORC activation.

Amino acid metabolism is intrinsically disturbed in fasted LCAD KO mice

To further address the role of disturbed amino acid metabolism in the origin of fasting-induced hypoglycemia, we stimulated mobilization of protein stores prior to food withdrawal by treating WT and LCAD KO mice with rapamycin, an mTORC inhibitor. Rapamycin decreased the levels of phosphorylated rpS6 in the liver and heart of fasted WT mice, showing that the treatment was effective (Fig. 5A). Rapamycin also decreased the levels of phosphorylated rpS6 in fasted LCAD KO mice. This further indicates that the elevated levels of phosphorylated rpS6 in fasted LCAD KO mice are related to increased mTORC signaling (Fig. 5A).

Despite efficiently decreasing the levels of phosphorylated rpS6, rapamycin did not rescue hypoglycemia in LCAD KO mice after 9 h (Fig. 5B) or an overnight period of fasting (Supplementary Material, Fig. S4C). Whereas only subtle changes in the amino acid profile were observed upon overnight fasting, rapamycin treatment caused prominent changes in the amino acid profile after 9 h of fasting. The total amino acid level in plasma increased consistent with protein mobilization, but this increase was more pronounced in WT animals (Fig. 5B). Also, the contribution of the different amino acids to the total pool was different. Whereas glutamine levels increased in both WT and LCAD KO mice, alanine increased only in WT animals (Fig. 5B). The plasma BCAA levels increased in both WT and LCAD KO animals upon rapamycin treatment, but remained much higher.
Figure 4. Failure to mobilize protein stores in fasted LCAD KO mice. (A) Blood glucose levels after L-alanine administration in overnight-fasted animals and the AUC for the normalized blood glucose with the initial blood glucose set to zero ($n=5$ for WT and $n=6$ for LCAD KO). Error bars indicate SEM. (B) Total liver protein content in postabsorptive and overnight-fasted WT and LCAD KO mice ($n=7$ for WT postabsorptive; $n=9$ for LCAD KO postabsorptive; $n=11$ for WT overnight fast; $n=11$ for LCAD KO overnight fast). Error bars indicate SEM. (C) Levels of hepatic ribosomal protein (rp)S6 and its phosphorylated (S235/236) form in overnight-fasted WT and LCAD KO mice. Error bars indicate SEM. (D) Levels of cardiac rpS6 and its phosphorylated (S235/236) form in overnight-fasted WT and LCAD KO mice. Error bars indicate SEM. (E) The fraction of BCAA as percentage of the total free amino acid pool and the glutamine levels in liver, skeletal muscle, heart and plasma. Data were derived from Supplementary Material, Tables S3–S5. Error bars indicate SEM.
in LCAD KO mice (Fig. 5B). These data suggest that the ALAT reaction in LCAD KO mice remains deficient despite stimulation of protein degradation. It further illustrates that due to the primary defect in FAO, amino acid metabolism is intrinsically disturbed in fasted LCAD KO mice with mTORC activation and impaired protein degradation as secondary events. This explains why rapamycin cannot rescue hypoglycemia in fasted LCAD KO mice.

DISCUSSION

Patients with an FAO defect are at risk for developing fasting-induced hypoglycemia. It is currently unknown why gluconeogenesis does not compensate for the increased glucose demand during fasting to maintain normoglycemia in these patients. We have now performed a detailed study of the major metabolic adaptations to fasting in the LCAD KO mouse. We...
show that, due to the defect in FAO, peripheral tissues utilized more glucose for the maintenance of energy homeostasis. This led to a rapid onset of hypoglycemia in fasting LCAD KO mice. In the initial state of fasting, the high glucose demand was met by accelerated hepatic glycogenolysis, likely driven by low hepatic G6P levels. However, as soon as glycogen stores were depleted, LCAD KO mice reached a steady state characterized by efficient peripheral glucose uptake and a slightly decreased EGP. At this stage, gluconeogenesis was similar in WT and LCAD KO mice. But, in order to restore normoglycemia, hepatic G6P levels and glycogen stores, gluconeogenesis should have been enhanced to a greater extent in LCAD KO mice than in WT mice. Unexpectedly, gluconeogenesis was not dysfunctional *per se*, but the failure of LCAD KO mice to adequately enhance gluconeogenesis was explained by limiting the availability of glucogenic precursors.

Targeted analysis of the plasma metabolome revealed decreased circulating levels of lactate, pyruvate and glucogenic amino acids such as alanine and glutamine in LCAD KO mice when compared with WT mice. Since gluconeogenesis was not increased in these animals, the low levels of glucogenic precursors cannot be explained by their accelerated use for gluconeogenesis. This was further evidenced by the similar levels of glycerol, a glucogenic precursor derived from lipolysis. Indeed, we provide evidence that the low circulating levels of glucogenic precursors were caused by a decreased mobilization of protein stores in liver and peripheral tissues.

Alanine and glutamine are not only glucogenic precursors, they also transport nitrogen derived from peripheral protein degradation to the liver for subsequent conversion into urea (24,25). Indeed, most of the alanine produced by muscle is derived from glucose via ALAT and is converted into glucose in the liver. This transport process is named the glucose–alanine cycle. We show that LCAD deficiency led to the suppression of this glucose–alanine cycle. Transaminases are thought to function close to equilibrium, thus relatively small changes in substrate concentration will change the net flux through these enzymes. In LCAD KO mice, increased pyruvate oxidation is needed to compensate for decreased acetyl-CoA production from fatty acids, leaving less pyruvate available for glutamate transamination via ALAT (Supplementary Material, Fig. S4D).

In addition, alanine production in muscle is tightly coupled to the degradation of BCAAs (24,25). As a consequence of a decreased alanine production, transamination of BCAAs will be decreased as well, leading to a decrease in glutamine levels and an increase in the contribution of BCAAs to the total amino acid level in tissues such as skeletal muscle and heart (Supplementary Material, Fig. S4D). BCAAs, notably leucine, but also glutamine, are regulators of mTORC signaling and as such promote protein synthesis and inhibit autophagy (21). Protein homeostasis was clearly disturbed in fasted LCAD KO mice, which was evidenced by an apparent failure to degrade proteins in the liver and heart, decreased levels of mono- and polyubiquitinated proteins in the liver and increased levels of phosphorylated rpS6 in the liver and heart. Pharmacological inhibition of mTORC by rapamycin in LCAD KO mice was unable to increase alanine levels to the same extent as in WT mice, suggesting that the ALAT reaction in LCAD KO mice remains deficient despite stimulation of protein degradation. It further illustrates that due to the primary defect in FAO, amino acid metabolism is intrinsically disturbed with mTORC activation and impaired protein degradation as secondary events. Based on these observations, we speculate that the physiologic role of FAO during fasting may not primarily be the sparing of glucose, but rather the sparing of pyruvate for transamination, enabling the glucose–alanine cycle and ultimately mobilization of protein stores. The importance of pyruvate-sparing for peripheral amino acid metabolism is also illustrated by the phenotype of Pdk4 KO mice. During fasting, Pdk4 KO mice are unable to appropriately inactivate pyruvate dehydrogenase, leading to low tissue pyruvate levels and as a consequence decreased alanine and increased BCAA levels (26). In the LCAD KO mouse, Pdk4 mRNA levels were increased in the liver (Table 1) in an apparent attempt to limit the conversion of pyruvate into acetyl-CoA.

The unexpected link between FAO, amino acid metabolism and mobilization of protein stores may not only explain the hypoglycemia in LCAD KO mice. It could also offer an alternative explanation for the cardiac hypertrophy observed in these animals. We have previously reported that fasting leads to the loss of cardiac mass in WT mice but not in LCAD KO mice (11). Hypertrophy associated with FAO defects may, therefore, not be solely related to cardiac energetics or lipotoxicity, but in addition could be caused by a shift in the protein balance. Indeed, we found elevated levels of phosphorylated rpS6 in hearts of LCAD KO mice. Therefore, novel therapeutic approaches should be aimed at restoring protein homeostasis. Inhibitors of mTORC, such as rapamycin, may be useful for this purpose. Alternatively, treatment with high-protein diets (27) or anaplerotic substrates such as odd-chain triglyceride has been proposed (28) and should be further explored.

Most of the changes in hepatic glucose metabolism of the fasted LCAD KO mouse can be explained by hypoglycemia and thus are directly linked to the increased glucose requirement in the periphery. Blood glucose levels drive glucokinase flux, because this flux is determined by the activity of two hepatic glucose sensors, Glut2 and glucokinase, which both have low affinity for glucose. The low glucokinase flux, on its turn, leads to low hepatic G6P levels, which cannot be compensated for by increased glycogen turnover or enhanced gluconeogenesis in LCAD KO mice. Similar to glucokinase, the activity of G6Pase is regulated by its substrate concentration because $K_{m}$ of the enzyme is higher than the intracellular concentration of G6P (29). Indeed, in a perifusion system containing rat hepatocytes, hepatocellular G6P levels have a virtual linear relationship with gluconeogenesis rate (30). It is noteworthy that several changes in hepatic glucose fluxes were paralleled by hepatic gene expression patterns. These changes in hepatic gene expression are consistent with decreased transcriptional regulation by insulin and carbohydrate response element-binding protein, which positively impact on Pdk4 expression and negatively on Slc2a2, Gck, Pygl2 and Pklr (31,32).

The results in the LCAD KO mouse resemble to some extent those obtained in a model of acute FAO inhibition, using the carnitine palmitoyltransferase 1 inhibitor 2-tetradecylglycicylic acid (33). Fasted 2-tetradecylglycicylic acid-treated mice are hypoglycemic, which is caused by an increased MCR of glucose while EGP remains unaffected. Importantly, 2-tetradecylglycicylic acid-treated mice do not enhance gluconeogenesis to counteract the hypoglycemia. Thus, acute FAO inhibition by pharmacological means as well as genetic FAO deficiency leads to hypoglycemia as a
consequence of increased glucose demand in peripheral tissues, which is not compensated for by enhanced gluconeogenesis.

Importantly, future research should be aimed at confirming the contribution of this connection between FAO and amino acid metabolism to hypoglycemia in human subjects with an FAO defect. In addition, we speculate that this connection not only plays a role in the pathogenesis of FAO defects, but is likely also relevant for other clinical conditions characterized by disturbed FAO, such as obesity. Indeed, a BCAA-related metabolite signature associates with incidence, progression and remission of insulin resistance and type 2 diabetes (34–36).

In conclusion, we show that a genetic defect in murine FAO leads to an increase in peripheral glucose requirement during fasting. This drives previously unrecognized changes in hepatic and peripheral carbon metabolism. Importantly, gluconeogenesis cannot compensate for the increased glucose demand in FAO-defective animals due to a shortage of gluconeogenic precursors. We suggest that FAO should not only be considered as a glucose-sparing process as classically defined by the Randle cycle. Rather, FAO is also a pyruvate-sparing process, which from a viewpoint of metabolic regulation is highly important given the central role of pyruvate in intermediary metabolism.

MATERIALS AND METHODS

Animals

Acadl+/− mice (B6.129S6-Acadl<sup>tm1UAB/Mmmh</sup>) (15) on a pure C57BL/6 background were obtained from Mutant Mouse Regional Resource Centers (http://www.mmrrc.org/). Regular backcrossing with C57BL/6N (Charles River) was performed to prevent genetic drift. Male LCAD KO and WT mice were generated in heterozygote as well as homozygote breeding pairs. pups were genotyped using PCR in combination with acetyl carnitine analysis on bloodspots. Mice used for this study were backcrossed for 13 to 16 generations and analyzed at age 2 to 6 months. During these experiments, mice were fed commercially available laboratory chow [CRM (E); Special Diets Services, Someren, The Netherlands]. The housing facility was kept at 21°C (±2°C) with a relative humidity of 55% (±10%). Rooms had a 12 h light (<60 lx):12 h dark cycle with a 0.5 h dim phase. For the postabsorptive state, mice were placed in a clean cage at 8 a.m. and killed and dissected 4 to 6 h later. For the overnight-fasted condition, mice were placed in a clean cage at 4 p.m. and fasted until dissection the next morning (16 to 20 h). Mice were anesthetized using ketamine (87 mg/kg) and xylazine (13 mg/kg) and terminated by blood collection via a catheter in the vena cava inferior. Multiple sampling of blood glucose levels was performed using a Contour glucometer (Bayer, Mijdrecht, The Netherlands). For this, blood was drawn from the tail vein or from the vena saphena. For a glucose challenge, overnight-fasted animals received GlucaGen (Novo Nordisk) at a dose of 48 μg/kg BW (i.p.). For the 1- alanine tolerance test, overnight-fasted animals received 500 mg/kg BW 1-alanine (i.p.).

For hepatic carbohydrate flux measurements, male LCAD KO mice and WT mice were housed in a temperature- and light-controlled facility and fed commercially available laboratory chow (RMH-B; Hope Farms, Woerden, The Netherlands). Mice were used for experimental procedures at 50–60 days of age. After the infusion experiment, mice were sacrificed by cardiac puncture under isoflurane anesthesia. All experiments were approved by the institutional review board for animal experiments at the University of Groningen or the Academic Medical Center, Amsterdam.

For rapamycin (LC Laboratories, Woburn, MA, USA) treatment, 5 mg of rapamycin was first dissolved in 0.2 ml ethanol, followed by adding 0.125 ml of sterile 10% PEG 300/8% ethanol and 0.125 ml of sterile 10% Tween 80 and then that solution was further diluted by adding 9.55 ml 0.9% NaCl. Using this cosolvent, rapamycin (0.5 mg/ml) remains soluble for several hours. Mice received a dose of 2 mg/kg i.p. prior to the onset of fasting. In the 9 h fasting experiment, mice were injected at 9 a.m., and blood was collected from the saphenous vein in non-anesthetized mice for glucose and amino acid profiling. For the overnight fast, mice were injected at 5 p.m. and sacrificed the next morning after pentobarbital anesthesia (100 mg/kg).

Blood and plasma metabolite determination

After collection, EDTA blood (75 μl) was mixed immediately with 75 μl of 1 M perchloric acid and stored on ice for at least 10 min. After centrifugation, the supernatant was neutralized using 2 M KOH, 0.5 M 2-(N-morpholino)ethanesulfonic acid (MES). Glucose, lactate, pyruvate and beta-hydroxybutyric acid (BHBA) concentrations were measured in the neutralized supernatant after the removal of KClO4 using established procedures (37). The remainder of the blood sample was used for plasma preparation. Plasma was used for the determination of free fatty acids (Wako Chemicals, Neuss, Germany), triglycerides (Human, Wiesbaden, Germany), glycerol (Randox, Crumlin, UK) and cholesterol (Ecoline, DiaSys Diagnostic Systems, Holzheim, Germany). Plasma amino acids and acylcarnitines were measured using UPLC tandem mass spectrometry (MS) and tandem MS, respectively (38,39).

Unsupervised hierarchical clustering was performed using complete linkage and Spearman rank correlation distance on the 44 normalized metabolites using software implemented in GenePattern (40–42). The z-score was calculated on the mean of each row. Two missing values for lysine were replaced by the row mean. Color in the heat maps reflects the relative metabolite abundance level, with red being higher and blue lower than the mean metabolite abundance value. Metabolite and animal ordering is determined as in hierarchical clustering, using the distance function 1-correlation. RF analysis of the metabolite data was performed using the MetaboAnalyst webserver (43).

The following features were added: total amino acids, total BCAA, %BCAA of total amino acids, BCAA/propionylcarnitine, BCAA/C5-carnitine, BCAA/C5OH-carnitine, BCAA/lysine, BCAA/glutamine, BCAA/alanine, C14:1-carnitine/acyethylcarnitine, C14:1-carnitine/free carnitine, free fatty acids/BHBA and lactate/pyruvate.

Hepatic carbohydrate flux measurements

Hepatic carbohydrate fluxes were determined in vivo using stable isotope infusion basically as described (44,45). Mice were equipped with a jugular vein catheter attached to the skull by acrylic glue under isoflurane anesthesia (46). Following the surgery, mice were allowed a recovery period of 5 days. Prior
to the infusion experiment, starting at 7 a.m., mice were fasted for 9 h. The infusion solution contained 7.0 μmol/ml [U-13C]-glucose, 81 μmol/ml [2,13C]-glycerol, 17 μmol/ml [1,2H]-galactose and 1 mg/ml paracetamol with an infusion rate of 0.6 ml/h (33). Blood and urine spots were collected at hourly intervals on filter paper (Schleicher and Schuell No. 2992, 's Hertogenbosch, The Netherlands). Additionally, blood glucose levels were measured every hour using EuroFlash™ test strips (LifeScan Benelux, Beerse, Belgium).

**Glucose derivatization and mass isotopomer distribution analysis**

Paracetamol-glucuronide (ParGlcUA) was extracted from urine spots on filter paper using methanol/water (3/1v/v). ParGlcUA was converted to its methyl ester, using a freshly made mixture of methanol and acetylcholoride (10/1v/v). Paracetamol-glucoside (ParGlc) was formed from the methyl ester by adding sodium borohydride and allowed to react overnight. ParGlc was isolated by an HPLC system (Waters, Etten Leur, The Netherlands) on a Nucleosil 7C18 SP250/10 column. Finally, ParGlc was hydrolyzed to paracetamol and glucose using β-glucosidase (44). Glucose was extracted from bloodspots using a mixture of water and ethanol (1/10v/v) (33,44). The isolated glucose of each plasma and urine sample was split into two parts and derivatized to its pentaacetate and aldonitril-pentaacetate esters, respectively (33,44). Samples were analyzed by gas chromatography (GC)-MS by positive ion channel ionization (45,47). The measured fractional isotopomer distribution by GC-MS (M₀–M₆) was corrected for the fractional distribution from natural abundance of 13C by multiple linear regression (48). This yields the excess fractional distribution of the mass isotopomers M₀–M₆ incorporated by infused labeled compounds. MIDA algorithms of isotope incorporation and dilution were used to determine hepatic carbohydrate fluxes (45,47).

**Tissue metabolite measurement**

Tissues were freeze-clamped directly after termination, using liquid nitrogen, or snap-frozen in liquid nitrogen rapidly. The frozen material was placed in liquid nitrogen, using a mortar and pestle or a BioPulverizer (Biospec Products, Bartlesville, OK, USA). For G6P and glycogen, powdered liver was extracted in 5% perchloric acid, using sonication (once at 8 W output, 80 J, on ice). After centrifugation, the supernatant was neutralized with 2 M KOH, 0.5 M MES and used for the determination of G6P (37). Glycogen was determined in this extract by digestion using amyloglucosidase (37). Hepatic glycogen content was expressed as glucose equivalent per milligram protein. For amino acid measurements, powdered tissue was extracted using sonication (once at 8 W output, 80 J, on ice) in 80% acetonitril containing internal standards. The supernatant was used for UPLC tandem MS analysis. Protein pellets were dissolved in 1 M KOH and used for protein determination and subsequent normalization.

**Deoxyglucose uptake**

Mice were fasted overnight and received 76 kBq 2-[14C(U)]-deoxy-d-glucose (Perkin Elmer) in 0.9% NaCl (i.p. 250 μl). Blood glucose and radioactivity were measured at 0, 20 and 40 min. Mice were killed by cervical dislocation at 40 min after which they were rapidly dissected and tissues snap-frozen in liquid nitrogen (white adipose tissue, heart, m. soleus, m. gastrocnemius, brown adipose tissue and brain). Tissues were homogenized in distilled water using an Ultra-Turrax after which the homogenate was heated to 95°C in a water bath for 30 min. After heating, the samples were centrifuged for 10 min at maximal speed. The supernatant was applied on an anion exchange column (Dowex, 1 × 8–200, Sigma) in order to separate phosphodeoxyglucose from deoxyglucose. Glucose uptake was calculated by dividing the uptake of phosphodeoxyglucose [in disintegrations per minute (dpm)/mg protein] by the AUC of the blood deoxyglucose label per blood glucose molecule (dpm/ nmol glucose) (49).

**Quantitative real-time RT–PCR analysis**

Total RNA was isolated from the liver, using Trizol (Invitrogen) extraction, after which cDNA was prepared using the SuperScript II Reverse Transcriptase kit (Invitrogen). Quantitative real-time PCR analysis of selected genes was performed in this cDNA using the LC480 Sybr Green I Master mix (Roche). Primer sequences are available upon request. To confirm the amplification of a single product, both melting curve and sequence analyses were carried out. All samples were analyzed in duplicate. Data were analyzed using linear regression analysis as described by Ramakers et al. (50). To compare the expression levels between different samples, values were normalized against the values for Rppl0 (36B4, ribosomal protein, large, P0).

**Enzyme measurements**

Tissue samples were disrupted using an Ultra-Turrax followed by sonication (twice at 8 W output, 40 J, on ice) in PBS. The homogenates were centrifuged for 5 min at 1000g. Protein concentration was determined in the supernatant, using the BCA method. Palmitoyl-CoA dehydrogenase activity was measured as described elsewhere using palmitoyl-CoA as substrate and ferricenium hexafluorophosphate as electron acceptor (51). The production of trans-2,3-palmitoyl-CoA and L-3-hydroxypalmitoyl-CoA was quantified by HPLC and used to calculate acyl-CoA dehydrogenase activity (52).

**Immunoblot analysis**

Freeze-clamped or rapidly frozen tissue was pulverized and extracted by sonication in PBS containing 0.1% Triton X-100, phosphatase inhibitor cocktail 2 and 3 (Sigma) and cOmplete, Mini Protease Inhibitor Cocktail (Roche). Phospho-S6 ribosomal protein (Ser235/Ser236) antibody (#2211) and S6 ribosomal protein (54D2) mouse monoclonal antibody (#2317) were obtained from Cell Signaling (Danvers, MA, USA). Mono- and polyubiquitylated conjugates were visualized using an HRP-conjugated mouse monoclonal antibody (clone FK2, Enzo Life Sciences). Antibodies were visualized using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

**Statistics**

Data are displayed as the mean ± the standard deviation (SD) or standard error of the mean (SEM) as indicated in the
ACKNOWLEDGEMENTS

The authors thank Theo Boer, Arno van Cruchten, Rick Havinga, Judith de Vos and the employees of the Animal Research Institute, Amsterdam, for technical assistance, and Vincent de Boer, Riekelt Houtkooper, Peter Voshol, Hans Waterham and members of the Laboratory Genetic Metabolic Diseases for helpful suggestions.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Netherlands Organization for Scientific Research (VIDI grant No. 016.086.336 to S.M.H.), the Tjallingh Roorda Foundation and Top Institute Food and Nutrition (TIFIN) grant (No. A102 to H.H.).

REFERENCES


