# Where does liver fat go? A possible molecular link between fatty liver and diabetes

Ectopic fat accumulation in insulin-target organs leads to the development of insulin resistance in each organ by altering oxidative stress[1,2] and gene expression profiles[3,4]. Specifically, the liver functions as a center to maintain whole-body energy homeostasis by sensing nutrient stimuli, and producing a variety of nutrients and bioactive substances. Liver fat is associated with not only enhanced hepatic glucose production, but also skeletal muscle insulin resistance, supporting a central role of fatty liver in systemic insulin resistance in patients with non-alcoholic fatty liver disease (NAFLD)[5]. Of the triacylglycerol accumulated in the liver of patients with NAFLD, 59.0% arises from non-esterified fatty acids (NEFAs), mainly derived from adipose tissue, whereas 14.9% arises from diet and 26.1% from *de novo* lipogenesis[6]. Triglyceride (TG) itself is not a toxic lipid, but might rather protect against the toxic effects of free fatty acids. However, some free fatty acids are regarded as toxic lipids that cause hepatic insulin resistance through oxidative stress[7]. In an *in vitro* fatty liver system using H4IIEC3 hepatocytes, a saturated fatty acid, palmitate, and not an unsaturated fatty acid, oleate, inhibits insulin-stimulated tyrosine phosphorylation of insulin receptor substrate 2 and serine phosphorylation of Akt through c-Jun $NH_2$-terminal kinase activation[8]. In that model, mitochondrial β oxidation-derived reactive oxygen species (ROS) play a causal role in the palmitate-induced c-Jun $NH_2$-terminal kinase activation[8]. Therefore, toxic lipid-induced mitochondrial ROS might underlie the link between steatosis and

insulin resistance in the liver. Indeed, in humans, genes involved in mitochondrial oxidative phosphorylation (OXPHOS)/electron transport chain are more coordinately upregulated and positively correlated with those involved in a ROS-related pathway in the livers of obese type 2 diabetic patients compared with those of non-obese type 2 diabetic patients[4]. These findings suggest that mitochondria play a key role in governing energy homeostasis in the liver, and might be a potential therapeutic target for type 2 diabetes and NAFLD.

Recently, Burgess *et al.*[9,10] proposed that an enhanced OXPHOS pathway in the liver causes elevated hepatic glucose production, oxidative stress and inflammation. They focused on substrate fluxes in the mitochondrial tricarboxylic acid (TCA) cycle and OXPHOS. Mitochondria play a central role in energy production in whole tissues, mostly in the form of adenosine triphosphate, through the TCA cycle. As the TCA cycle is the major source of electrons for adenosine triphosphate production, a small percentage of electrons can 'leak' from the electron transport chain, composed of respiratory chain complex I–IV, to induce the formation of ROS, even during normal electron transport. For liver in an overnutrition state, lipid and glucose are insufficiently oxidized, leading to a decrease in the adenosine triphosphate production/oxygen consumption ratio, which leads to further ROS production in the mitochondria. Such excess ROS production can contribute to further hepatic mitochondrial dysfunction, and the development of hepatic insulin resistance and NAFLD. In addition to excess ROS formation, genetic factors, aging and reduced mitochondrial biogenesis also contribute to further mitochondrial dysfunction. Mitochondrial function in hepatic metabolic processes involves

anaplerosis and cataplerosis pathways. The pathways are metabolic processes that allow 4-carbon intermediates to move into (anaplerosis) and out of (cataplerosis) the TCA cycle. Anaplerosis and cataplerosis pathways should operate at the same flux to avoid accumulation of the intermediates in the TCA cycle (Figure 1).

Sunny *et al.*[9] quantitated intrahepatic TG content (IHTG) using $^1H$ magnetic resonance spectroscopy, and assayed anaplerotic substrate flux and cataplerotic substrate efflux in the mitochondrial TCA cycle by a tracer method using $^2H$ and $^{13}C$ in participants with NAFLD. Hepatic oxidative flux in the TCA cycle was doubly increased in participants with high IHTG compared with those with low IHTG; 50% higher rates of lipolysis increased mitochondrial anaplerosis, which increased cataplerotic efflux, leading to 30% higher rates of gluconeogenesis. There was a strong positive correlation between IHTG content and anaplerotic flux. Unexpectedly, ketone production assessed by tracer dilution of β-hydroxybutylate was not different between participants with high and low IHTG, suggesting a relatively suppressed ketogenic pathway even under enhanced TCA cycle flux in human NAFLD.

Next, Satapati *et al.*[10] examined the molecular mechanisms underlying increased hepatic anaplerotic flux in diet-induced mouse and rat models of obesity. In concert with the aforementioned human data, ketogenesis, oxidative flux in the TCA cycle, anaplerosis, cataplerosis and gluconeogenesis were all increased in the livers from fed mice perfused with high NEFA (0.8 mmol/L) compared with those perfused with low NEFA (0.2 mmol/L). Although insulin administration to the liver perfused with high NEFA suppressed glycogenolysis, it did not prevent the elevation in anaplerosis and gluconeogenesis. Therefore, NEFA

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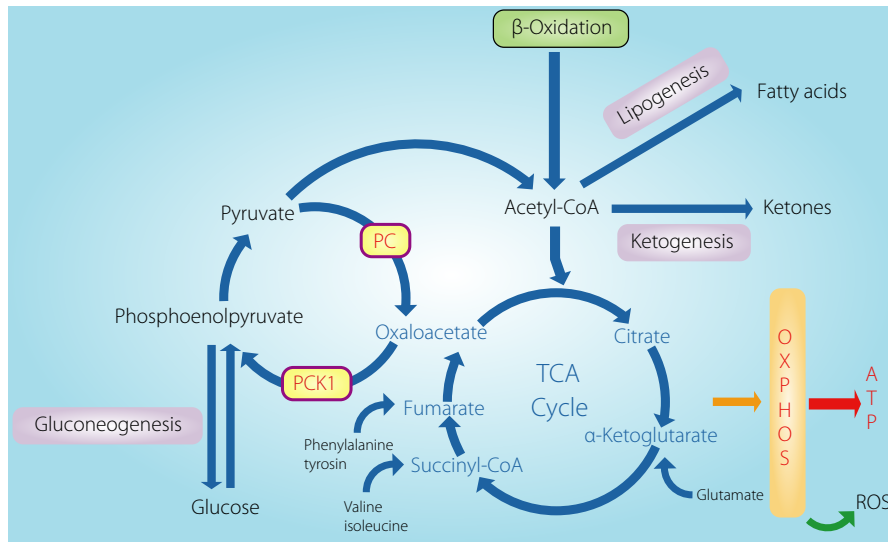E-mail address: ttakamura@m-kanazawa.jp

**Figure 1** | Schematic of the hepatic tricarboxylic acid (TCA) cycle with the major anaplerotic and cataplerotic pathways associated with glucogenonesis, lipogenesis, and ketogeneis. An elevation of fatty acid delivery amplifies the TCA cycle flux with a rise in anaplerosis/cataplerosis, leading to a proportional rise in oxidative stress and inflammation in liver. The redox state and intermetabolites in the TCA cycle amplify or suppress the flux. Oxidative stress is causal for further mitochondrial dysfunction. ATP, adenosine triphosphate; OXPHOS, oxidative phosphorylation; PC, pyruvate carboxylase; PCK1, phosphoenolpyruvate carboxykinase; ROS, reactive oxygen species.

overload is considered sufficient to increase *ex vivo* hepatic oxidative metabolism, anaplerosis and gluconeogenesis. In agreement with the *ex vivo* liver perfusion, in the liver from rats in which circulating NEFA was increased by infusing intralipid for 6 h, ketone turnover, oxidative flux in the TCA cycle, and gluconeogenesis were increased together with increased circulating levels of TG and insulin. Thus, increased lipid delivery is sufficient to amplify anaplerosis and gluconeogenesis *in vivo*. In that *in vivo* model, messenger ribonucleic acid levels for mitochondrial superoxide dismutase 2, inflammatory cytokines, such as *tnfa* and *Il6*, and lipid peroxidation were elevated in proportion to the rise in oxygen consumption that is strongly associated with anaplerosis/cataplerosis flux and gluconeogenesis during intralipid infusion.

In an *in vitro* study using H4IIE hepatocytes[10], NEFA increased ROS production, which was attenuated with an inhibitor for phosphoenolpyruvate carboxykinase (PEPCK), which links cataplerosis efflux from the TCA cycle and gluconeogenesis. Based on this *in vitro* finding, they investigated the therapeutic role of PEPCK in NAFLD *in vivo*. Systemic knockdown of *Pck1* (PEPCK-C) retained the ability to suppress hepatic glucose production during a hyperinsulinemic–euglycemic clamp study. Also, insulin-induced phosphorylation of Akt was reduced in the liver of mice fed a high fat diet (HFD), which was rescued by *Pck1* knockdown. Interestingly, *Pck1* knockdown sufficiently prevented a fatty acid-induced rise in anaplerosis/cataplerosis in mice fed a HFD, but not in mice fed a control diet. Why does *Pck1* knockdown decrease anaplerosis/cataplerosis? TCA cycle oxidation is regulated by redox states and product inhibition[11]. *Pck1* knockdown reduced the hepatic mitochondrial and cytosolic nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide ratio estimated from the plasma acetoacetate/β-hydroxybutylate and liver lactate/pyruvate ratio, respectively. Because nicotinamide adenine dinucleotide serves as a coenzyme for dehydrogenases in the TCA

cycle, a reduced nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide ratio suppresses TCA flux. Such a redox state is known to inhibit the forward reaction of the TCA cycle[11]. In addition, several TCA cycle metabolites are known to inhibit upstream enzymes, which is known as product inhibition[11]. *Pck1* knockdown markedly increased oxaloacetate, one of the intermediate products produced during the TCA cycle, which inhibits upstream enzyme succinate dehydrogenase. As the TCA cycle is the major source of electrons for OXPHOS, a *Pck1* knockdown-mediated decrease in anaplerosis/cataplerosis might decrease ROS production through OXPHOS. Indeed, hepatic expression of the genes involved in oxidative stress, peroxidase and peroxiredoxins were upregulated by a HFD, which was canceled by *Pck1* knockdown. Also, HFD-induced lipid peroxidation was canceled by *Pck1* knockdown. *Pck1* knockdown resulted in a more oxidized redox state of mitochondrial inner membranes. Furthermore, a reduced cytosolic redox state is favorable for the reduced form of glutathione and the clearance of peroxide by its anti-oxidant system. *Pck1* knockdown accumulated some intermediates in the TCA cycle that activate anti-oxidant transcription factors, such as nuclear respiratory factor 1 and nuclear factor, erythroid-derived 2, like 2. Collectively, *Pck1* knockdown reduces electron transport and activates anti-oxidant systems, and thus reduces ROS levels in the liver. In agreement with low oxidative stress, *Pck1* knockdown also protected mice against HFD-induced inflammation.

Metformin suppresses hepatic glucose production, and is used as a first-line oral antidiabetic agent for type 2 diabetes. Metformin might prevent a rise in anaplerosis/cataplerosis, as it suppresses *Pck1* messenger ribonucleic acid levels in a concentration- and time-dependent manner[12]. Indeed, Satapati *et al.*[10] showed that metformin treatment reduces fasting hepatic gluconeogenesis by suppressing TCA flux and anaplorosis/cataplorosis in mice fed a HFD. Also, metformin reduced the

hepatic expression of *Tnfa* and *IL6* expressions in mice fed a HFD.

Finally, Satapati *et al.*[10] examined the significance of oxidative metabolism in human NAFLD pathology. The histological NAFLD activity score of the human liver biopsy specimens correlated with oxygen consumption, indicating that activation of oxidative metabolism is associated with liver pathology in human NAFLD.

The Burgess *et al.*[9,10] linked mitochondrial oxidative metabolism, including amplified TCA cycle flux and anaplorosis/cataplerosis, to hepatic oxidative stress, inflammation and/or insulin resistance in mice infused with NEFA. Notably, substrate flux into ketogenesis is differently regulated in humans[9] and mice[10]. How substrate flux is regulated still remains unclear. Metformin protects mice fed a HFD from oxidative stress and inflammation by reducing mitochondrial oxidative metabolism[10]. However, in a clinical setting, metformin seems unsatisfactory in ameliorating human NAFLD pathology[13]. Instead, they have proposed another candidate therapeutic target for reducing mitochondrial oxidative metabolism. Unexpectedly, inhibition of PEPCK protects HFD-fed mice from oxidative stress and inflammation by reducing mitochondrial oxidative metabolism. As anaplerosis is elevated in people with NAFLD[9], further studies are required to provide evidence of whether a PEPCK inhibitor is effective in ameliorating the pathology of NAFLD, including liver histology and insulin resistance, in humans.

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