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The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes

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Abstract

Non-alcoholic fatty liver disease and its downstream sequelae, hepatic insulin resistance and type 2 diabetes, are rapidly growing epidemics, which lead to increased morbidity and mortality rates, and soaring health-care costs. Developing interventions requires a comprehensive understanding of the mechanisms by which excess hepatic lipid develops and causes hepatic insulin resistance and type 2 diabetes. Proposed mechanisms implicate various lipid species, inflammatory signalling and other cellular modifications. Studies in mice and humans have elucidated a key role for hepatic diacylglycerol activation of protein kinase Cε in triggering hepatic insulin resistance. Therapeutic approaches based on this mechanism could alleviate the related epidemics of non-alcoholic fatty liver disease and type 2 diabetes.

Modern global health care faces challenges that are drastically different from past generations, largely owing to the increasing worldwide prevalence of obesity. This is exemplified by a change in focus to centre on obesity-related liver disease. Although viral hepatitis continues to be an important health concern, non-alcoholic fatty liver disease (NAFLD) is the now most common liver disorder in the Western world, where the rates of adult and paediatric obesity have soared to an estimated 20–30% of the US population[1,2]. In east and south Asian communities, NAFLD is also on the rise, with estimates that its prevalence reaches as high as 60% in urban areas[3,4]. Startlingly, NAFLD has been found to be highly prevalent among young lean south Asian Indians[5,6].

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A strong association between NAFLD and type 2 diabetes has been demonstrated: more than 90% of obese patients with type 2 diabetes have NAFLD[7]. Insulin resistance is common in both conditions[5]. Patients with NAFLD almost universally have hepatic insulin resistance, which increases the risk of impaired fasting glucose and type 2 diabetes[5,8–11]. In addition, a subset of patients with NAFLD will develop non-alcoholic steatohepatitis (NASH) with histological changes such as steatosis, lobular inflammation and/or hepatocellular ballooning[12]. Around 20% of patients with NASH will progress to liver cirrhosis and liver failure[13,14]. NASH-associated cirrhosis is now the third most common indication for liver transplantation in the United States[15]. Health policies that can prevent NAFLD and new treatments that can reverse the disease will offer tremendous benefits, in terms of both lives saved and health-care costs.

Thus, in this Perspective we will discuss the link between hepatic lipid accumulation and hepatic insulin resistance and focus on the role of diacylglycerol, a lipid metabolite that activates novel protein kinase C iso-forms (PKCs) and thereby impairs insulin signalling, in the pathogenesis of lipid-induced hepatic insulin resistance. Although several other mechanisms have been proposed to explain this association, these alternatives have been reviewed elsewhere[16]. As we will discuss here, diacylglycerol-induced novel PKC activation has emerged as a common mechanism to explain the development of insulin resistance in liver and skeletal muscle in a variety of experimental and clinical models.

Molecular mechanism of lipid-induced insulin resistance

Insulin action requires a coordinated, intricate relay of intracellular signals, involving mostly phosphorylation and dephosphorylation events. In the canonical view of hepatic insulin signalling, insulin binds and activates the insulin receptor tyrosine kinase (IRTK), which in turn promotes tyrosine kinase phosphorylation of insulin receptor substrates (IRS), most importantly IRS2 in the liver (Fig. 1)[17]. Phosphorylation of IRS2 generates binding sites for Src homology 2 domain proteins, including phosphatidylinositol-3-OH kinase (PI(3)K)[18]. The binding of PI(3)K to IRS2 recruits phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3), which in turn recruits Akt[19]. Under insulin-stimulated conditions, 3-phosphoinositide-dependent kinase-1 phosphorylates and activates Akt, which is thought to suppress hepatic glucose production through two key mechanisms: first, decreased expression of gluconeogenic enzymes by phosphorylation and nuclear exclusion of the fork-head box protein FOXO1 and its pro-gluconeogenic targets, and second, activation of glycogen synthase by phosphorylation and inactivation of glycogen synthase kinase-3β. Although this relatively linear construct is useful for interrogating insulin signalling in experimental models, it fails to capture the interwoven mechanisms that have evolved to regulate hepatic glucose and lipid metabolism. For example, although acute insulin signalling following a meal can decrease messenger RNA expression of gluconeogenic enzymes, it probably does not acutely alter the protein levels of these enzymes. Gluconeogenic enzymes are also conventionally thought to be subject to allosteric activation: acetyl coenzyme A (acetyl-CoA) activates pyruvate carboxylase[20,21], and fructose-2,6-bisphosphate inhibits fructose-1,6-bisphosphatase[22]. And, although insulin might activate glycogen synthesis, glucose is necessary to inhibit glycogen phosphorylase and effectively promote net hepatic glycogen synthesis[23,24].

The development of NAFLD is strongly associated with hepatic insulin resistance. This relationship is most apparent when NAFLD is induced in rats after just 3 days of being fed a high-fat diet. The ability of insulin to suppress hepatic glucose production is diminished in this model even without changes in body weight, adiposity or muscle insulin resistance[25]. Hepatic insulin resistance in this model was associated with increased hepatic diacylglycerol content and increased translocation of the primary novel PKC isoform in liver, protein kinase-Cε (PKCε)[26,27], to the plasma membrane at which it was found to bind and inhibit the activity of the intracellular kinase domain of the insulin receptor. This was associated with reduced insulin-stimulated phosphorylation of IRS2 and IRS2-associated PI(3)K activity and phosphorylation of Akt2. Consequently, the ability of insulin to activate glycogen synthesis and inhibit gluconeogenesis was impaired (Fig. 1)[25]. The crucial role of PKCε in mediating lipid-induced hepatic insulin resistance has been convincingly demonstrated by knocking down expression of PKCε in the liver of rodents. Rats treated with an antisense oligonucleotide (ASO) to decrease hepatic expression of PKCε were protected from lipid-induced hepatic insulin resistance, although hepatic diacylglycerol and triglyceride content were unchanged[26]. Furthermore, these animals were found to have preserved IRTK activity with intact signalling through downstream proteins. Similarly, *Prkce* whole-body knockout mice are also protected from lipid-induced insulin resistance[28].

This model for lipid-induced hepatic insulin resistance has been translated to humans. Potential mechanisms for hepatic insulin resistance were assessed in a group of patients undergoing bariatric surgery. Although the participants were all obese, there was a significant variation in insulin resistance. Notably, individuals with very similar body mass index could manifest markedly different degrees of insulin resistance. By contrast, hepatic diacylglycerol content and PKCε activation were the strongest predictors of hepatic insulin resistance in liver biopsies obtained from these individuals[29]. There was no association between insulin sensitivity and other factors implicated in causing hepatic insulin resistance, including ceramide content, endoplasmic reticulum (ER) stress markers or inflammatory cytokine concentrations. These results were replicated in another study showing that hepatic diacylglycerol content was the best predictor of hepatic insulin resistance in obese humans, whereas there was no association with hepatic ceramide content or markers of inflammation[30]. Indeed, hepatic inflammation has been suggested to be a consequence, not a cause, of insulin resistance[31]. Thus, although excess calorie intake certainly leads to obesity, only those who develop hepatic steatosis will develop insulin resistance. These data argue that the key step in the pathogenesis of hepatic insulin resistance is the accumulation of hepatic diacylglycerol leading to activation of PKCε. Experimental models of altered hepatic lipid content allow us to further test this mechanism and could also inform the development of potential therapeutic interventions.

The model outlined focuses on the inability of insulin to alter hepatic glucose metabolism. Despite this, the ability of insulin to activate lipogenesis seems to be intact in most models of NAFLD. Much has been written about the paradox of selective insulin resistance, with investigators proposing the existence of branch points in the insulin signalling pathway[32,33], but space limitations preclude discussion of selective insulin resistance in this Perspective.

Instead, we will discuss the mechanisms that govern hepatic lipid accumulation and its relationship to insulin's ability to control hepatic glucose metabolism.

Regulation of fat delivery to liver

Hepatic lipid content is regulated by the balance between hepatic lipid uptake, synthesis, oxidation and export (Fig. 2). Hepatic lipid uptake is a function of substrate delivery and transport into the hepatocyte, and several genetic models exemplify this aspect of hepatic lipid metabolism. Transgenic mice with liver-specific overexpression of lipoprotein lipase (LpL) develop liver-specific lipid accumulation and liver-specific insulin resistance, whereas transgenic mice with muscle-specific overexpression of LpL develop muscle-specific lipid accumulation and muscle-specific insulin resistance[34]. In these models, tissue-specific insulin resistance followed ectopic lipid accumulation. In a similar example, mice that lack the primary fatty-acid transporter in hepatocytes, FATP5, are protected from diet-induced NAFLD, indicating that excess fatty-acid transport into the hepatocyte is required for NAFLD and hepatic insulin resistance[35].

Studies in mice and humans have implicated adipose tissue lipolysis as an important source of fatty acids that promote NAFLD and hepatic insulin resistance. Whole-body lipolysis increases with total fat mass in humans[36,37]; however, the relationship between lipolysis and insulin sensitivity seems to be largely independent of body mass. For example, insulin-resistant obese adolescents have higher visceral fat content than their weight-matched, insulin-sensitive counterparts[38]. The relationship between adipose lipolysis and hepatic lipid content is exemplified by manipulation of the genes that regulate adipose lipolysis. As in humans, the effect of lipolysis on insulin sensitivity in rodents seems to be independent of body weight. Obese mice lacking the fatty-acid-binding protein FABP in adipocytes are more insulin sensitive than their obese littermates with normal FABP[39]. Conversely, leptin-deficient obesity-prone mice with increased rates of adipocyte lipolysis due to knockout of the gene encoding adipocyte phospholipase A2 show increases in ectopic lipid storage and insulin resistance despite reduced body weight compared with littermates with normal lipolytic rates[40]. These data suggest a facilitative role for the increases in adipose tissue lipolysis in providing substrates for ectopic lipid deposition and insulin resistance.

These genetic rodent models inform our understanding of human disease. Patients with conditions resulting from mutations in LpL (for example, hyperlipoproteinaemia type 1) are prone to developing insulin resistance[41]. In humans, the single nucleotide polymorphism (SNP) rs56225452, putatively representing a gain-of-function mutation in the FATP5 promoter, was associated with insulin resistance and NAFLD[42]. In Asian Indian individuals, as well as those of other ethnic groups, variants (C-482T, T-455C or both) in apolipoprotein C3 (APOC3), which can inhibit LpL and hepatic lipase, are associated with hypertriglyceridaemia and NAFLD[43]. These polymorphisms led to around 30% higher plasma APOC3 concentrations and post-prandial hypertriglyceridaemia through the inhibitory effect of APOC3 on LpL activity. As a result, the livers of individuals with APOC3 variants take up a greater amount of lipid from chylomicrons, remnants of lipoprotein particles, predisposing these lean subjects to NAFLD and hepatic insulin resistance. These results were replicated in another cohort of lean males of European

descent[44]. Of note, this *APOC3*-gene–environment interaction has only been observed in lean males, probably reflecting a protective effect of oestradiol on the ability of APOC3 to inhibit LpL and promote ectopic fat storage[45], and the ability of obesity-associated NAFLD[44] to mask the relatively subtle affect of this gene–environment interaction on the development of hepatic insulin resistance[46,47].

The effect of increased plasma Apoc3 concentrations on the development of NAFLD and hepatic insulin resistance has been genetically validated in transgenic mice that have increased hepatic overexpression of Apoc3. These mice are more prone to diet-induced NAFLD and diacylglycerol–PKCε-induced hepatic insulin resistance than their wild-type littermates[48]. Interestingly, although hypertriglyceridaemia was present in transgenic mice fed both a normal and high-fat diet, severe hepatic steatosis and hepatic insulin resistance only developed in Apoc3 transgenic mice fed a high-fat diet, reflecting an important gene–environment interaction. Moreover, the phenotype was due to both inhibition of peripheral lipase activity and diminished hepatic triacylglycerol export as very-low-density lipoprotein (VLDL)[48].

Lessons learned from lipodystrophy

The importance of adipose tissue lipid storage is exemplified when adipose tissue is altogether absent. In ‘fatless’ mice expressing the dominant-negative protein A-ZIP/F-1 in adipocytes, the absence of visceral and peripheral fat leads to ectopic lipid accumulation and severe hepatic and muscle insulin resistance. Insulin resistance can be corrected by transplantation of white adipose tissue from normal mice, further illustrating the importance of adipose tissue as a ‘safe’ storage depot[49]. Lipoatrophic mice with the gene encoding peroxisome proliferator-activated receptor-γ (Ppar-γ) knocked out in white adipose tissue or with the gene encoding hepatic 1-acylglycerol-3-phosphate O-acyltransferase 2 (Agpat2) knocked out globally display a similar phenotype: the loss of visceral and subcutaneous fat is associated with hepatic steatosis and hepatic insulin resistance[50,51].

Similar associations are evident in humans with lipodystrophies. Patients with these disorders represent a rare example of severe hepatic insulin associated with extreme hepatic steatosis in the absence of visceral or peripheral fat accumulation[52–54]. Leptin treatment can decrease calorie intake and effectively normalize hepatic lipid content and hepatic insulin action[53,54]. Similarly, patients with partial lipodystrophy owing to mutations in the scaffolding protein perilipin-1, which inhibits adipose triglyceride lipase, have reduced peripheral fat mass but develop NAFLD because of increased adipose tissue lipolysis resulting from inhibition of adipose tissue triglyceride lipase[55]. These patients can also develop profound insulin resistance. These data again point to ectopic lipid accumulation in the liver, which might occur as a result of the diversion of substrates from other storage depots, as the crucial mediator of hepatic insulin resistance.

Regulation of hepatic lipid synthesis

Hepatic triacylglycerol synthesis is the sum of two main processes: the synthesis of fatty acids (*de novo* lipogenesis, DNL) and esterification of fatty acids into fatty-acid glyceride species (for example, mono-, di- and triacylglyceride).

Contributions of *de novo* lipogenesis to triacylglycerol synthesis

Although DNL is thought to make a relatively small contribution to hepatic triacylglycerol accumulation relative to esterification[56,57], rates of postprandial DNL do increase significantly in both young and elderly patients with NAFLD[58–60]. Diet-induced NALFD might stimulate a feed-forward loop exacerbating DNL and ectopic lipid deposition. For example, fructose-fed hamsters have hypertriglyceridaemia, NAFLD and insulin resistance associated with increased DNL[61], and because fructose inhibits fatty-acid oxidation both directly and indirectly, excess fructose intake is likely to stimulate DNL and hepatic insulin resistance[62,63]. However, reducing lipogenic gene expression by knockdown of the upstream gene encoding peroxisome proliferator-activated receptor γ coactivator-1β (PGC-1β)[64] protects against fructose-induced hepatic insulin resistance[65]. Similarly knockdown of the genes encoding the acetyl CoA carboxylases ACC1 and ACC2, which are crucial in the regulation of DNL and lipid oxidation, respectively, reduced liver triacylglycerol and diacylglycerol content, decreased PKCε activation and protected mice from lipid-induced hepatic insulin resistance[66].

Skeletal muscle insulin resistance promotes hepatic lipogenesis

Skeletal muscle insulin resistance typically accompanies insulin resistance at other sites, possibly because of the diversion of substrates from insulin-resistant muscle to the liver (Fig. 3). The independent effect of muscle insulin resistance to exacerbate NAFLD has also been demonstrated in rodents. Mice that lack insulin-responsive glucose transporter 4 (Glut4) in muscle have NAFLD[67]. Similarly, mice lacking the Akt substrate As160 have decreased glucose uptake in adipose tissue and slow-twitch muscles, and develop insulin resistance in the liver as well as adipose tissue and skeletal muscle[68]. Muscle insulin resistance might also be independently associated with NAFLD. For example, the severity of NAFLD in mice fed a high-fat, high-cholesterol diet was demonstrated to correlate with peripheral insulin resistance, and not with hepatic insulin resistance[69].

These results have been translated to humans, in which selective muscle insulin resistance in healthy young lean individuals has been shown to predispose them to increased hepatic DNL, hepatic triacylglycerol accumulation and atherogenic dyslipidaemia after eating high-carbohydrate meals. This is because ingested glucose is diverted away from muscle glycogen storage to the liver, in which it is converted to triacylglycerol driven by the compensatory hyperinsulinaemia that is secondary to muscle insulin resistance[57]. Further evidence to support this hypothesis stems from a study demonstrating that a single 45 minute bout of exercise on an elliptical trainer increased postprandial muscle glycogen synthesis following carbohydrate ingestion, resulting in a 40% reduction in hepatic DNL and a 30% reduction in hepatic triglyceride synthesis[70].

Fatty acid esterification contributes to triacylglycerol synthesis

Most liver triglyceride is formed through esterification of fatty acids[56,57]. Diacylglycerol is an intermediate in the esterification pathway and, thus, genetic models that manipulate hepatic lipid esterification can be used to further examine the diacylglycerol–novel-PKCs hypothesis of insulin resistance. Rats overexpressing the rate-controlling enzyme in triglyceride esterification, mitochondrial glycerol-3-phosphate acyltransferase 1 (GPAT1),

have hepatic insulin resistance associated with increased PKCε activity[71]. These data are in contrast to mice in which the gene encoding mitochondrial GPAT was knocked down. These animals exhibit suppression of PKCε activity and improved hepatic insulin sensitivity[72], offering further evidence in support of the diacylglycerol–PKCε hypothesis of hepatic insulin resistance.

Diacylglycerol acyltransferase 2 (DGAT2) catalyses the final step in triglyceride synthesis from diacylglycerol. Although inhibition of DGAT2 may be expected to acutely increase cellular diacylglycerol content, chronic reduction in hepatic DGAT2 expression due to ASO treatment results in decreased hepatic diacylglycerol content due to downregulation of the lipogenic pathway. Consistent with the diacylglycerol–PKCε hypothesis this reduction in hepatic diacylglycerol content was associated with reduced PKCε activation and protection from lipid-mediated hepatic insulin resistance[73,74].

The effects of hepatic overexpression of DGAT2 are less clear. Monetti *et al*. reported that although transgenic mice had increased hepatic diacylglycerol content, there was no impact on hepatic insulin sensitivity[75]. Jornayvaz *et al*. also demonstrated an increase in hepatic diacylglycerol content in the same mice, but reported increased PKCε activation, decreased hepatic insulin signalling and hepatic insulin resistance[76]. Technical differences in study execution could explain this difference. In hyperinsulinaemic–euglycaemic clamp studies to test insulin resistance, both groups found that DGAT2 transgenic mice fed a normal diet failed to normally suppress hepatic glucose production. However, the teams reported differing results for wild-type mice fed a normal diet. Jornayvaz *et al*. showed that these control mice had normal insulin suppression of hepatic glucose production, whereas Monetti *et al*. reported that control mice did not suppress hepatic glucose production. Thus, the key to interpreting the phenotype attributed to increased hepatic DGAT2 expression and diacylglycerol accumulation in this model is whether or not the control animals fed a normal diet had complete suppression of hepatic glucose production in response to insulin. In a different study in humans, SNPs in DGAT2 predicted a smaller decrease in liver fat content after very modest (3 kg) weight loss compared with individuals without SNPs in DGAT2. However, insulin signalling was not measured in these studies, and the participants' very modest weight loss may prevent us from uncovering meaningful information about the role of DGAT2 (ref. 77). Further studies are needed to understand the role of DGAT2 in hepatic insulin sensitivity and its potential link to whole-body adiposity.

Lipid export

Export of triglyceride as VLDL is the only means of reducing hepatic lipid content other than through fat oxidation. NAFLD-associated insulin resistance is seen in genetic models of impaired VLDL export[78], whereas, in models of increased VLDL export, NAFLD is ameliorated, independent of body weight[79,80]. As previously discussed, impaired hepatic lipid export also contributes to the development of NAFLD and hepatic insulin resistance in mice overexpressing ApoC3 (ref. 48).

Regulation of hepatic lipid oxidation

Rodent models with altered fat oxidation can be used to both test relationships between diacylglycerol, PKCε activation and insulin resistance, and to validate potential therapeutic targets. Mice lacking Ppar-α, a key regulator of hepatic lipid oxidation, are prone to NAFLD and fail to benefit from the insulin sensitizing effects of omega-3 fatty acids[81]. Mice that are genetically deficient in the dehydrogenase LCAD have diminished mitochondrial fatty-acid oxidation and increased *de novo* diacylglycerol synthesis, increased liver PKCε activity and hepatic insulin resistance[82]. Interestingly, humans and mice with loss-of-function mutations in LCAD exhibit fasting hypoglycaemia, which has been ascribed to defects in amino-acid metabolism and may not be related to alterations in hepatic insulin sensitivity[83]. Hypoglycaemia has also been dissociated from insulin resistance in mice fed a ketogenic diet. These mice develop profound hepatic insulin resistance associated with increased diacylglycerol concentrations and PKCε activation, despite hypoglycaemia stemming from reductions in fasting hepatic gluconeogenesis[84].

Changes in whole-body energy metabolism also affect hepatic lipid balance. Thyroid hormone receptor-α knockout mice have increased energy expenditure (as well as decreased expression of lipogenic enzymes) and are protected from diet-induced NAFLD, PKCε activation and hepatic insulin resistance[85]. Similarly, infusion of fibroblast growth factor 21 (Fgf21) in mice resulted in increased energy expenditure in the liver and white adipose tissue, lowered liver diacylglycerol concentrations, decreased hepatic PKCε translocation and protection from lipid-induced hepatic insulin resistance[86]. By contrast, mice deficient for fatty acid amide hydrolase (FAAH) have reduced whole-body energy expenditure due to diminished hypothalamic–pituitary axis activity and impaired thyroid function. The absence of FAAH resulted in increased liver diacylglycerol, increased PKCε translocation and hepatic insulin resistance[87]. These data again indicate that the link between NAFLD and insulin resistance is an increase in diacylglycerol concentration leading to activation of PKCε activity.

As already mentioned, inhibition of ACC1 and ACC2 leads to both a decrease in lipid synthesis and an increase in lipid oxidation. The latter effect is due to the disinhibition of carnitine palmitoyltransferase-1 (CPT1) and more long-chain fatty acyl CoAs entering the mitochondria. Mutation of key serine residues in Acc1 and Acc2 in mice prevents inactivation of these enzymes by AMP-activated protein kinase (AMPK), leading to increased lipid synthesis and decreased lipid oxidation[88]. These mice have increased hepatic diacylglycerol content, activation of PKCε and develop hepatic insulin resistance. By contrast, AMPK is activated in mice with a deletion of the mitochondrial gene encoding the sodium-dicarboxylate cotransporter Slc13a5, the mammalian homologue of the INDY protein in *Drosophila*. The increased AMPK activity leads to reductions in Acc1 and Acc2 activity, resulting in decreased liver diacylglycerol content and reduced PKCε translocation. Together, these changes protected *Slc13a5* knockout mice from both diet- and age-associated hepatic insulin resistance[89].

Many groups have studied whether people with NAFLD have alterations in hepatic oxidative flux either as a cause or consequence of ectopic lipid accumulation. Results in

humans have been mixed. When an indirect tracer method was used to assess hepatic tricarboxylic acid (TCA) cycle flux and anaplerosis, both were found to be markedly increased in individuals with NAFLD[90,91]. But when a ^{31}P magnetic resonance spectroscopy technique was used to assess hepatic ATP production, a reduction in hepatic energy metabolism was found in individuals with type 2 diabetes[92,93]. Because the role of hepatic oxidative flux is key to both the understanding of pathogenesis and potential treatment of NAFLD and type 2 diabetes, the development of methods to directly measure rates hepatic oxidative metabolism is of crucial importance[94].

Dissociation of NAFLD and hepatic insulin resistance

The requirement of NAFLD for hepatic insulin resistance has been questioned by several groups and has been recently reviewed[95–97]. It is well established that it is possible to experimentally induce insulin resistance without NAFLD, or induce NAFLD without insulin resistance under certain conditions. For instance, blocking hepatic VLDL secretion with a choline-deficient diet or by genetic modification of the export machinery increases hepatic triglyceride concentrations but does not cause insulin resistance[98,99]. Similarly, mice with liver-specific knockout of the gene encoding the protein phosphatase Shp1 develop NAFLD, but are protected from insulin resistance[100]. Hepatic carbohydrate responsive element-binding protein and sterol regulatory element-binding protein-1 have recently been independently implicated in dissociating NAFLD and insulin resistance in mice and humans[101,102]. In addition, mice with the gene encoding the lipase activator CGI-58 knocked down have profound hepatic steatosis due to suppression of hepatic triglyceride lipolysis. However, these mice are protected against high-fat-diet-induced hepatic insulin resistance[103]. To explain this paradox, the authors of one study examined the subcellular localization of diacylglycerols in liver cells and found that knockdown of the gene encoding CGI-58 promoted diacylglycerol accumulation in lipid droplets, while protecting against diacylglycerol accumulation in the cell membrane and preventing PKCε translocation to the cell membrane[104]. These results are consistent with earlier studies in humans, also showing that compartmentation of diacylglycerols in the cytosolic[29] and membrane[105] compartments could be an important factor in the pathogenesis of liver and muscle insulin resistance. The different results between diacylglycerol accumulation in the membrane and the cytosolic compartments might reflect differences in the measurement and fractionating or lipid-extraction techniques used in the different studies and/or in the length of fasting. Nevertheless, the CGI-58 ASO data clearly indicate that lipids sequestered in lipid droplets do not promote PKCε activation and hepatic insulin resistance, and that this compartmentation of diacylglycerols and triacylglycerols in lipid droplets may explain other models of NAFLD that are not associated with hepatic insulin resistance, as discussed later. Future work will better discern the importance of specific lipid compartments in the pathogenesis of insulin resistance.

Similarly, liver-specific *Hdac3* knockout mice are prone to developing hepatic steatosis when fed both normal and high-fat diets and have been shown to be protected from lipid-induced hepatic resistance[106]. This apparent disconnect is probably also due to altered partitioning of diacylglycerols to the lipid droplet. Loss of Hdac3 in mice was associated with an increase in the lipid droplet protein Plin2 and decreased activation of PKCε. When

Plin2 expression was normalized with a specific ASO, the improvements in glucose tolerance were no longer evident. Taken together these data strongly suggest that diacylglycerols need access to a particular subcellular compartment to inhibit IRTK activity, whereas diacylglycerol accumulation in the lipid droplet is protective and does not lead to inhibition of IRTK activity and insulin resistance.

Genetic variants in the triglyceride hydrolase PNPLA3 (also known as adiponutrin) have been proposed to disassociate NAFLD from insulin resistance in normal weight and obese humans[46]. However, this conclusion was based on fasting plasma glucose and insulin measurements, and hepatic insulin responsiveness was not directly assessed in this study. Furthermore, because many of the subjects in this study were obese and relatively insulin resistant it is difficult to determine if PNPLA3 could exacerbate hepatic insulin resistance in these individuals. Additional insights into the role of PNPLA3 have been gained using an ASO to decrease expression of PNPLA3 in rats[107]. Reduced expression of PNPLA3 decreased hepatic lipid content owing to decreased lipid esterification, pointing to the potential duality of PNPLA3's functions in both triglyceride synthesis and hydrolysis[108]. Similarly, in obese Taiwanese children, variants (which had previously been identified[109] to strongly correlate with NAFLD in adults of European ancestry who were not all obese) did not correlate with NAFLD, but again this may be due to the obfuscating effects of obesity in this cohort; however, there was a significant association between glucokinase regulatory protein (GCKR) and NAFLD[110].

Reversal of NAFLD ameliorates hepatic insulin resistance

The most effective intervention to reverse NAFLD and hepatic insulin resistance in humans is weight loss. Hepatic steatosis quickly resolves in both obese patients with type 2 diabetes and non-diabetic normal weight individuals with NAFLD after a hypocaloric diet and a modest weight loss of less than 10% of total body weight[111]. This is accompanied by resolution of hepatic steatosis and normalization of fasting plasma glucose concentrations, hepatic glucose production and hepatic insulin sensitivity[111,112]. However, recidivism following weight loss is extremely common: less than 50% of those who have lost more than 10% of their body weight are able to maintain the weight loss after 1 year[113], and after 5 years, less than 25% have maintained their weight[114]. Nevertheless, improvements in insulin sensitivity and NAFLD can persist for 2 years after weight loss in overweight and obese individuals even after the weight is regained, arguing that there is a dissociation between obesity and insulin sensitivity or NAFLD[115].

In regards to potential medical therapies to treat NAFLD, thiazolidinediones, which are potent PPAR-γ activators, have been shown to lead to significant reductions in liver-fat content and improvements in hepatic insulin sensitivity[116–120]. This effect probably occurs by thiazolidinedione activation of PPAR-γ in subcutaneous fat tissue leading to increased insulin sensitivity and suppression of lipolysis, resulting in a redistribution of liver fat to the subcutaneous fat cells[116].

Increasing hepatic mitochondrial fat oxidation by promoting subtle increases in mitochondrial uncoupling could be another therapeutic target for NAFLD, hepatic insulin

resistance and type 2 diabetes. Promoting mitochondrial uncoupling by treating rats fed a high-fat diet with low doses of the mitochondrial protonophore 2,4-dinitrophenol (DNP) was shown to reduce hepatic triglyceride content, increase insulin-stimulated IRS2 tyrosine phosphorylation and PI(3)K activity, and improve insulin-mediated suppression of hepatic glucose production[25]. Consistent with these findings, simply reversing hepatic steatosis using a liver-targeted DNP to increase hepatic mitochondrial fat oxidation by about 60% was found to reverse hypertriglyceridaemia, hepatic and peripheral insulin resistance, and hyperglycaemia in rat models of NAFLD and type 2 diabetes. Furthermore, correction of liver and muscle insulin resistance was associated with marked reductions in diacylglycerol-PKCε and diacylglycerol-PKCθ activity in liver and muscle, respectively; and occurred independently of any changes in body weight, inflammatory mediators, FGF21, adiponectin or liver ceramide content[121]. Moreover, liver-targeting DNP resulted in a 50-fold increase in the ratio of toxic to effective doses compared with DNP. Taken together these data support the key role of diacylglycerol activation of novel PKC in mediating liver insulin resistance and demonstrate the potential feasibility of dissociating the toxic effects of DNP from its beneficial effects to promote subtle increases in hepatic mitochondrial uncoupling and hepatic fat oxidation to treat the related epidemics of NAFLD and type 2 diabetes.

Outlook

Hepatic insulin resistance is a complex phenomenon. Although many questions regarding the nature of the insulin-signalling defect remain unanswered, hepatic insulin resistance is almost universally associated with increases in hepatic triacylglycerol and diacylglycerol concentrations, with the latter leading to PKCε activation and subsequent inhibition of IRTK activity. The diacylglycerol-PKCε hypothesis of hepatic insulin resistance has recently been validated in humans with NAFLD. Exceptions to this rule can be attributed to compartmentation of diacylglycerols to the lipid droplet, in which it does not lead to inhibition of IRTK activity. NAFLD occurs when lipid supply to the liver exceeds rates of lipid oxidation and lipid export. Thus therapies targeted to reduce fatty-acid delivery to the liver, suppress diacylglycerol production, or raise mitochondrial fat oxidation by promoting subtle increases in hepatic mitochondrial uncoupling are of great interest to ameliorate NAFLD and type 2 diabetes.

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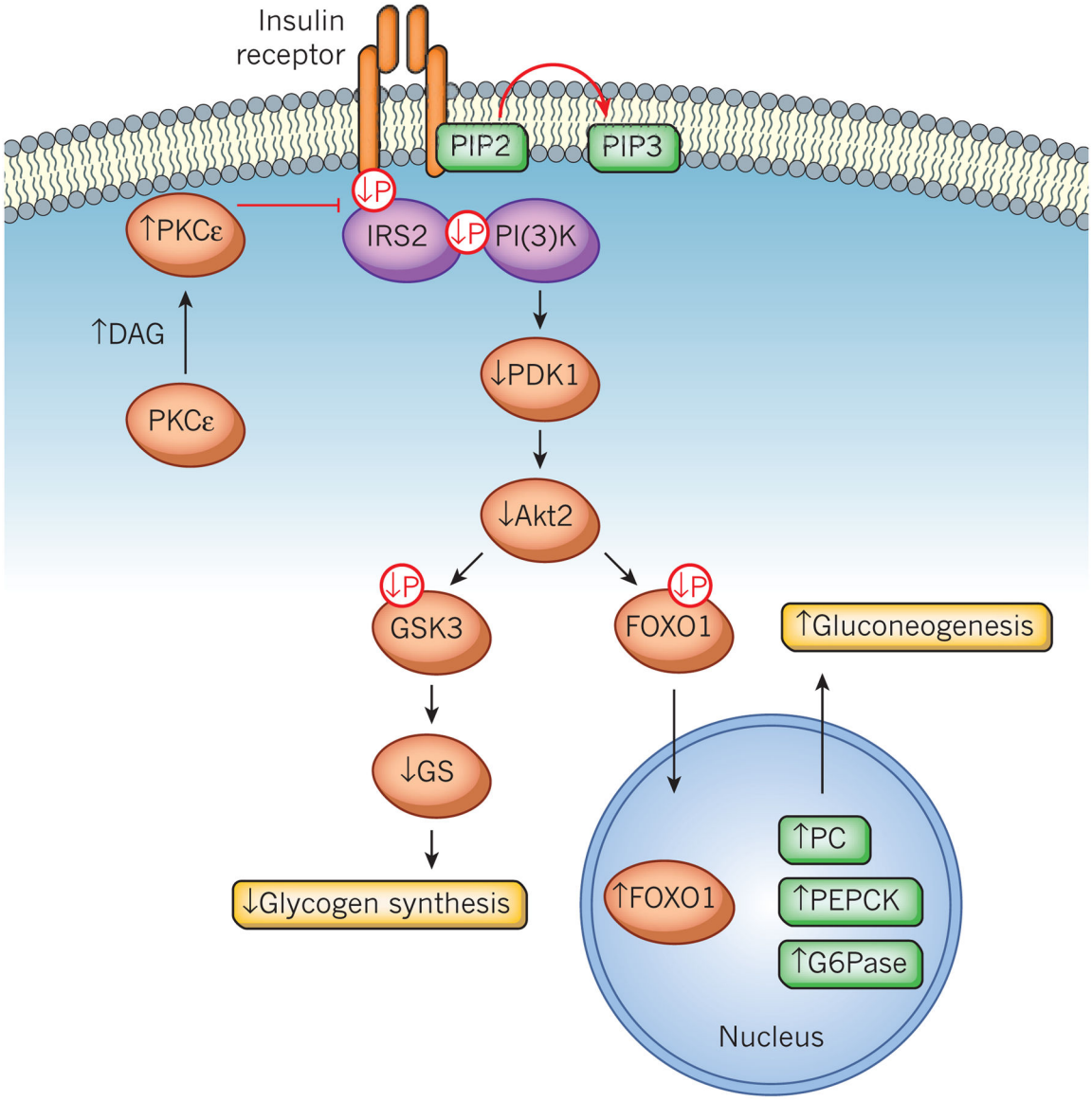

Figure 1. Molecular mechanism by which excess diacylglycerol leads to hepatic insulin resistance and hyperglycaemia

Increases in liver diacylglycerol (DAG) cause protein kinase Cε (PKCε) activation and translocation to the cell membrane, which results in inhibition of insulin signalling. Reduced phosphorylation of insulin receptor substrate-2 (IRS2) and PI(3)K impairs Akt2 activity by reductions in 3-phosphoinositide-dependent protein kinase 1 (PDK1) activity, suppressing glycogen synthase kinase-3 (GSK3) phosphorylation and reducing insulin-stimulated liver glycogen synthesis through reduced glycogen synthase (GS) activity. Impaired Akt2 activity also reduces insulin suppression of hepatic gluconeogenesis by promoting Forkhead box protein O1 (FOXO1) translocation to the nucleus due to reduced phosphorylation and increasing expression of the gluconeogenic proteins pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase). PIP3, phosphatidylinositol (3,4,5)-triphosphate.

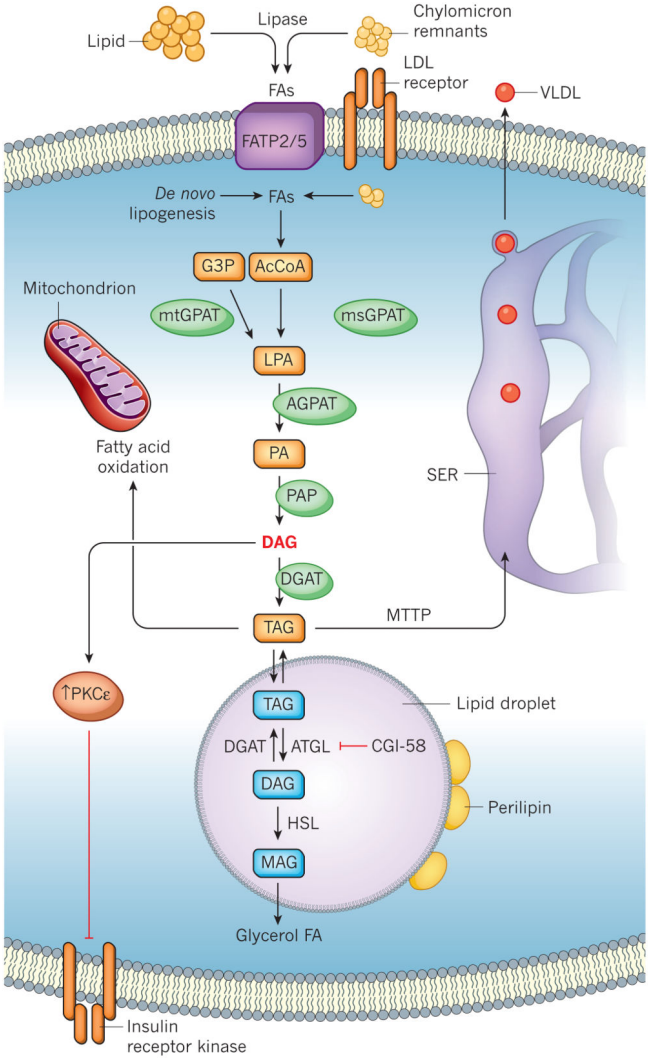

Figure 2. NAFLD develops due to an imbalance between lipid supply and demand

Fatty acids (FAs) derived from lipolysis and from chylomicron remnants are taken up through fatty-acid transport proteins (FATPs), mainly FATP2 and FATP5 in the liver; chylomicron remnants are also taken up through the low-density lipoprotein (LDL) receptor. A small fraction of intracellular fatty acid supply in the liver also comes from *de novo* lipogenesis in the cytosol. Fatty acids can also be re-esterified to lysophosphatidic acid (LPA) by acyl-coenzyme A (AcCoA) and the conversion of glycerol 3-phosphate (G3P) by either mitochondrial glycerol-3-phosphate acyltransferase (mtGPAT) or microsomal GPAT (msGPAT). Fatty-acyl CoAs (shown here as phosphatidic acid, PA) formed by 1-acylglycerol-3-phosphate O-acyltransferase-2 (AGPAT2) are then added to the glycerol backbone by phosphatidic acid phosphatase (PAP) to generate diacylglycerol (DAG), and by diacylglycerol acyltransferases (DGAT) to generate triacylglycerol (TAG). Increased DAG causes protein kinase Cε (PKCε) translocation to the cell membrane, which inhibits insulin signalling. Lipids may also be sequestered in lipid droplets as monoacylglycerol (MAG), DAG and TAG, but these are not thought to be responsible for hepatic insulin resistance. By inhibition of adipose triglyceride lipase (ATGL), comparative gene identification-58

(CGI-58) bound to perilipin is mainly responsible for lipid sequestration in the droplet. By contrast, intracellular hepatic lipid content is reduced by two mechanisms: mitochondrial fatty acid oxidation and export from the smooth endoplasmic reticulum (SER) as very-low-density lipoprotein (VLDL). HSL, hormone-sensitive lipase; MTTP, microsomal triglyceride transfer protein.

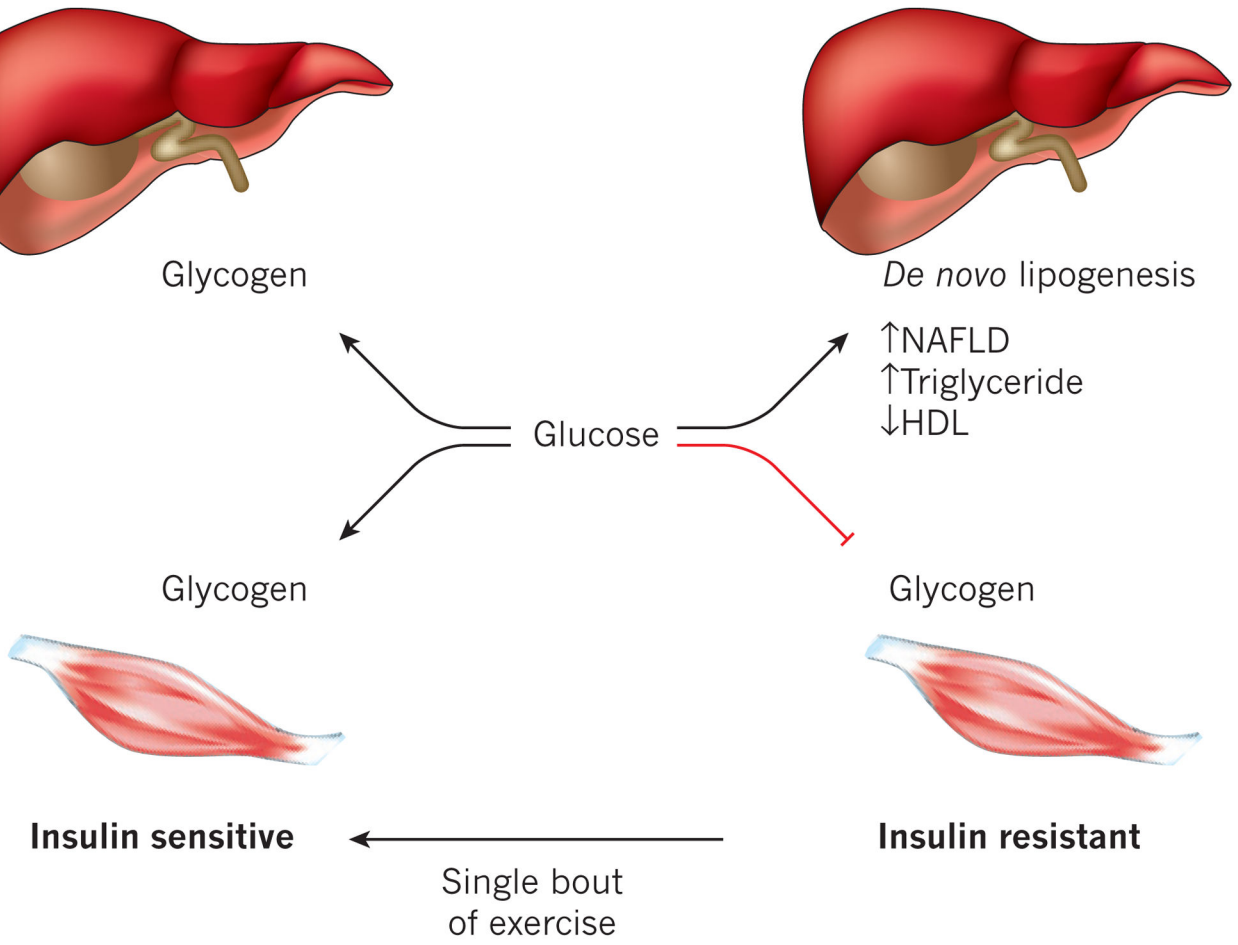

Figure 3. Mechanism by which selective skeletal muscle insulin resistance contributes to hepatic insulin resistance

In insulin-sensitive subjects, insulin stimulates glycogen synthesis in both liver and muscle; however, in those with skeletal muscle insulin resistance, insulin fails to promote glycogen synthesis, diverting substrate to *de novo* lipogenesis. Increased lipid synthesis in patients with muscle insulin resistance thus produces non-alcoholic fatty liver disease (NAFLD), with increased triglyceride and reduced high-density lipoprotein (HDL) export from the liver. However, these defects in muscle insulin signalling can be reversed by a single 45 minute bout of exercise.