The Liver as an Endocrine Organ—Linking NAFLD and Insulin Resistance

Matthew J. Watt,[1] Paula M. Miotto,[1] William De Nardo,[1] and Magdalene K. Montgomery[1]

[1]Department of Physiology, University of Melbourne, Melbourne, Victoria 3010, Australia

ORCiD numbers: 0000-0003-4064-4188 (M. J. Watt).

ABSTRACT The liver is a dynamic organ that plays critical roles in many physiological processes, including the regulation of systemic glucose and lipid metabolism. Dysfunctional hepatic lipid metabolism is a cause of nonalcoholic fatty liver disease (NAFLD), the most common chronic liver disorder worldwide, and is closely associated with insulin resistance and type 2 diabetes. Through the use of advanced mass spectrometry "omics" approaches and detailed experimentation in cells, mice, and humans, we now understand that the liver secretes a wide array of proteins, metabolites, and noncoding RNAs (miRNAs) and that many of these secreted factors exert powerful effects on metabolic processes both in the liver and in peripheral tissues. In this review, we summarize the rapidly evolving field of "hepatokine" biology with a particular focus on delineating previously unappreciated communication between the liver and other tissues in the body. We describe the NAFLD-induced changes in secretion of liver proteins, lipids, other metabolites, and miRNAs, and how these molecules alter metabolism in liver, muscle, adipose tissue, and pancreas to induce insulin resistance. We also synthesize the limited information that indicates that extracellular vesicles, and in particular exosomes, may be an important mechanism for intertissue communication in normal physiology and in promoting metabolic dysregulation in NAFLD. ***(Endocrine Reviews 40: 1367 – 1393, 2019)***

Nonalcoholic fatty liver disease (NAFLD) is defined by the accumulation of fat in the liver, in the absence of excessive alcohol consumption and other causes of hepatic steatosis, and encompasses a spectrum of conditions. Hepatic steatosis is also known as nonalcoholic fatty liver (NAFL) and is clinically characterized by the presence of visible lipid droplets containing triglycerides in >5% of hepatocytes when thin sections are assessed by light microscopy (1, 2), or a threshold of >5.56% when using proton magnetic resonance spectroscopy (3). Liver lipid levels are regulated by the interplay between the delivery of lipids to the liver and their hepatic uptake, synthesis, oxidation, and secretion within very low–density lipoproteins (VLDLs). Alterations in the equilibrium of one or more of these processes can promote hepatic steatosis (4). NAFL can further progress to nonalcoholic steatohepatitis (NASH), which is defined by hepatocyte ballooning, necrosis near steatotic hepatocytes, and mild inflammation, with or without different stages of fibrosis (5). Further progression of NASH can lead to life-threatening conditions such as cirrhosis, hepatocellular carcinoma, and terminal liver failure. Approximately 30% of adults in industrialized countries have NAFLD, and the global epidemic of obesity is driving a dramatic increase of NAFLD that is forecasted to result in increased clinical and economic burden (6, 7).

Hepatic steatosis/NAFL or early stage NAFLD is often described as a "benign condition" in the context of liver disease; however, the effects of steatosis extend beyond the liver. There are strong epidemiological links between NAFLD and type 2 diabetes, and steatosis is strongly associated with insulin resistance in the liver, and also in peripheral tissues such as skeletal muscle and adipose tissue (8–11). A major focus of current research is understanding the pathogenic mechanisms linking these comorbidities, and in this review, we focus on the impact of NAFLD on altering the endocrine function of the liver, and how the secretion of proteins, metabolites, and nucleic acids contributes to the pathophysiology of insulin resistance.

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ESSENTIAL POINTS

- Nonalcoholic fatty liver disease (NAFLD) is characterized by excessive lipid accumulation in hepatocytes and is the most common chronic liver disorder worldwide, affecting ~25% to 30% of adults in industrialized countries
- NAFLD is strongly associated with metabolic comorbidities, including obesity, type 2 diabetes, and dyslipidemia
- The liver secretes proteins, metabolites, and noncoding RNA that act as autocrine/paracrine and endocrine factors to influence metabolism in other tissues
- "Hepatokines" exert pleiotropic effects on lipid and glucose metabolism, as well as insulin action, and their secretion is impacted by NAFLD
- Liver-derived lipids and metabolites can serve as signaling molecules to regulate insulin action and other metabolic processes
- Liver-derived miRNAs may regulate glycemic control in NAFLD; however, definitive evidence is lacking

NAFLD and Diabetes

Prevalence

Concurrent with the increased global prevalence of obesity (12, 13), NAFLD has emerged as the most common chronic liver disorder worldwide, affecting ~25% to 30% of adults in industrialized countries, with ~20% of these NAFLD cases classified as NASH (6, 7). Importantly, NAFLD prevalence is forecasted to increase by 21% in the next 15 years, from 83.1 to 100.9 million individuals worldwide, with coincident increases in NASH and liver-related deaths (6). Liver-related deaths are likely due to the development of fibrosis (14), which occurs in ~35% of NAFLD patients (15) and is a major clinical concern.

NAFLD and insulin resistance

NAFLD is strongly associated with metabolic comorbidities, including obesity, type 2 diabetes, and dyslipidemia. Steatosis prevalence is increased in cohorts with obesity undergoing bariatric surgery, ranging from 70% to 96% (16–18), with NASH identified in 12% to 17% (16–18). Type 2 diabetes mellitus is closely associated with NAFLD, with more than three-fourths of type 2 diabetes patients reportedly having NAFLD (19–22).

Hepatic steatosis is epidemiologically associated with insulin resistance (19, 23–25). Results from small cross-sectional studies using gold-standard measures of insulin action have consistently shown that hepatic steatosis, independent of adiposity, is associated with impaired insulin action in liver, skeletal muscle, and adipose tissue in both lean individuals and nondiabetic individuals with obesity (8–11). Moreover, relatively small increases in liver fat are associated with hepatic and skeletal muscle insulin resistance, and further accumulation of liver fat beyond this relatively low threshold (~1.5% for liver insulin resistance and ~6% for muscle insulin resistance) is not associated with more severe insulin resistance (26). Thus, in contrast to the widely held idea that hepatic steatosis is a benign condition, it is now clear that steatosis is closely linked to impaired insulin action and type 2 diabetes, and it is an early predictor of metabolic disorders, particularly in the normal-weight population (27, 28). Moreover, hepatic steatosis precedes the development of skeletal muscle lipid accumulation, macrophage-related inflammation, hepatic, skeletal muscle, and adipose tissue insulin resistance, and whole-body hyperglycemia and hyperinsulinemia (29–31) in mice fed a high-fat diet. Taken together, these observations are consistent with the notion that changes occurring in the fatty liver alter paracrine and endocrine functions to cause insulin resistance in key glucoregulatory tissues.

Although hepatic steatosis is closely associated with systemic insulin resistance, it is noteworthy that insulin resistance also predicts the development of NAFLD. This results primarily from an impaired ability of insulin to suppress adipose tissue lipolysis, leading to increased delivery of free fatty acids to the liver (8, 10), and from increased *de novo* lipogenesis (32) that results from stimulation of lipogenic enzymes via sterol receptor–binding protein 1c (SREBP-1c), even in an insulin-resistant state (33). There is also evidence that triglyceride synthesis is increased through the Kennedy pathway (34) and that β-oxidation of fatty acids is decreased in insulin resistance, although the latter is controversial (35–37).

Although the association between NAFLD and insulin resistance in generally clear in most patients, this does not always hold true for specific genetically determined forms of fatty liver. For example, a frequent sequence variation (I148M) in patatin-like phospholipase domain–containing protein 3 (PNPLA3) is strongly associated with fatty liver disease in the absence of insulin resistance or dyslipidemia (38–40), and similar dissociations are reported in individuals with a single nucleotide polymorphism for acyl-coenzyme A (CoA):diacylglycerol acyltransferase

(41) and the Lys167 allele in transmembrane 6 superfamily 2 (TM6SF2) (42). In fact, the TM6SF2 actually protects from cardiovascular disease, despite high liver fat content and high prevalence for progression to NASH and cirrhosis (43). These observations highlight the need to understand the mechanistic bases of NAFLD for predicting the development of comorbidities and applicability of specific therapeutic interventions.

Liver Endocrine Function

Protein secretion—the role of "hepatokines"

The role of liver as a major secretory organ has long been appreciated, particularly with respect to its roles in regulating coagulation and hemostasis, but only recently has the potential magnitude for protein secretion become apparent. Mass spectrometry–based quantitative proteomics of human liver has quantified ~10,200 proteins (44), which parallels observations in mice (45). Given that ~40% of the transcripts in liver encode secreted proteins (46), there is clearly a large scope for significant and varied protein secretion from the liver.

The liver is composed of parenchymal cells, including hepatocytes and bile duct cells, which occupy ~80% of the liver volume, and nonparenchymal cells, such as sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells. Although there are some differences in the proteins expressed in these different cell types, the vast majority of liver proteins are expressed in all liver cell types, and the hepatocyte proteome constitutes the vast majority of the total liver proteome, indicating that hepatocytes are quantitively the most important cell type for liver protein secretion (45).

Several anatomical, structural, and functional features support the notion that the liver is an important organ for intertissue communication. The liver is large (~1.5 kg) and receives ~25% of the cardiac output, providing a substantial volume of blood and thereby secreted factors for redistribution to other tissues. In this context, the liver has unique architecture and blood flow regulation, whereby hepatocytes and nonparenchymal cells secrete products into the liver sinusoids, which flow via the central veins to the inferior vena cava and eventually to the heart for redistribution to peripheral tissues. The extensive vascular network, and particularly the "open pore" sinusoids that are located between hepatocyte planes, also supports the likelihood that hepatokines are prominent in paracrine and autocrine regulation of hepatocyte function.

Classical protein secretion

Classical or conventional secretion involves the transport of newly synthesized proteins through the organelles of the secretory pathway, including the endoplasmic reticulum (ER), the ER exit sites, the Golgi, and eventually to the plasma membrane via secretory vesicles or secretory granules for delivery of transmembrane proteins to the plasma membrane and soluble proteins to the extracellular space. Proteins secreted in this manner contain a signal peptide on the N terminus and most often contain posttranslational modifications that occur at the ER and Golgi (*i.e.*, glycosylation) (47).

Nonclassical protein secretion

Proteins that do not contain an N terminus signal peptide can be secreted as cargo contained within extracellular vesicles. There are three main types of extracellular vesicles that differ in terms of size, type of biogenesis, and composition. Exosomes are the smallest vesicle, ranging from ~40 to 100 nm in diameter, and are formed within multivesicular bodies that fuse to the plasma membrane and are released into the circulation as exosomes. Microvesicles such as ectosomes and endosomes range from 50 to 1000 nm and are formed by direct budding from the plasma membrane, whereas apoptotic bodies range from 2000 to 5000 nm and are secreted as a byproduct of cell death (48). These vesicles contain protein, lipid, and nucleic acid cargo that somewhat reflects their cell of origin, and the components of extracellular vesicles can be rapidly altered in response to metabolic challenges (49), highlighting their potential role in regulating intertissue communication and metabolism.

Hepatokines, NAFLD, and insulin resistance

Studies examining hepatocytes isolated from healthy and steatotic mouse livers have used quantitative proteomics to show that liver steatosis alters hepatokine secretion and that the protein signals originating from the steatotic liver alter fatty acid metabolism and induce inflammation and insulin resistance in other cell types. This section outlines the classically secreted hepatokines involved in regulating lipid metabolism and insulin action, and it describes the effect of NAFLD in these relationships [see Table 1 and Fig. 1 for hepatokine changes in the presence of NAFLD (50–86)]. We close this section with a brief description of the literature pertaining to liver exosome proteins and metabolism.

Activin E

Activin E is a member of the TGFβ family and is encoded by the inhibin βE gene (87). Activin E is a newly identified hepatokine (51) that is elevated in liver and serum in humans with obesity (88) and NAFLD (89). Mice that overexpress activin E gain less fat and have improved glucose tolerance when compared with wild-type mice fed a high-fat diet. This appears to be mediated by an increase in uncoupled respiration as evidenced by increased expression of thermogenic proteins in adipose tissue and higher core temperature in activin E overexpressing mice (51), as well as an inability of activin E knockout mice to

Table 1. Hepatokine Links With NAFLD and Insulin Resistance

Hepatokine	Gene (Mouse / Human)	Molecular Mass (kDa)	Expression in NAFLD	Contribution to NAFLD	Contribution to Glucose Tolerance/Insulin Resistance
Activin E	*Inhbe/INHBE*	~22[a]	Increased	Reduces steatosis (50)	Activin E overexpression prevents diet-induced glucose intolerance in rodents (51), unknown in humans
Adropin	*Enho/ENHO*	5	Decreased	Suppresses lipogenesis (52)	Improves insulin sensitivity (52); stimulates insulin signaling in skeletal muscle (53)
ANGPTL4	*Angptl4/ANGPTL4*	45	Increased	Promotes hepatic lipid accumulation (54–56)	Controversial findings: (i) improves insulin sensitivity (55, 56) by decreasing hepatic glucose output (55); (ii) causes insulin resistance in liver, skeletal muscle, and adipose (57, 58)
DPP4	*Dpp4/DPP4*	~30	Increased	Increases liver CD36 (59), likely to increase lipid storage	Inhibits incretin levels and impairs insulin secretion (60)
Ectodysplasin	*EDA*	~46	Increased	Unknown	Induces insulin resistance in skeletal muscle via JNK activation (61)
Fetuin A	*Ashg/ASHG*	~67	Increased	Unknown	Promotes insulin resistance in liver via ER stress and JNK activation (62); inhibits insulin receptor in skeletal muscle (63); ligand for TLR4, which promotes lipid-mediated insulin resistance in adipose tissue (64)
Fetuin B	*Fetub/FETUB*	~60	Increased	Unknown	Promotes insulin resistance in myocytes/hepatocytes (65); impairs whole-body glucose tolerance (65)
FGF21	*Fgf21/FGF21*	~23	Increased	Increases hepatic fat oxidation and decreases lipids (66); decreases adipose tissue lipolysis, reducing lipid availability to the liver (67)	Improves insulin sensitivity, decreases diacylglycerol (68); promotes insulin secretion of pancreatic β-cells (69)
Follistatin	*Fst/FST*	38	Increased	Promotes IL-1β production, may promote fibrosis development (70)	Unknown
HFREP1	*Fgl1/FGL1*	36	Increased	Promotes NAFLD (71); increases lipogenesis through ERK1/2 activation (72)	Causes insulin resistance in skeletal muscle via JNK activation (72)
HMGB1	*Hmgb1/HMGB1*	30	Increased	Unresolved; blocking HMGB1 protects against NAFLD (73, 74)	Unknown; however, HMGB1 activates TLR4 and causes inflammation in hepatocytes that could impair insulin sensitivity (75)
Inhibin βE	*Inbe*	39	Increased with obesity	Unknown	Unknown
LECT2	*Lect2/LECT2*	16	Increased	Unknown	Promotes insulin resistance in skeletal muscle via JNK activation (76); impairs insulin signaling via serine/threonine phosphorylation of IRS1 (77)
PEDF	*Serpinf1/SERPINF1*	50	Increased	Interacts with ATGL to increase lipolysis; ablation promotes steatosis (78, 79).	Promotes insulin resistance in skeletal muscle via JNK activation (80).
RBP4	*Rbp4/RBP4*	21	Increased	Unknown	Equivocal. Overexpression in liver does not alter glucose homeostasis or insulin sensitivity (81); whole-body overexpression causes insulin resistance by activating JNK and TLR4 (82)
SeP	*Selenop/SELENOP*	~60	Increased	Unknown	Causes insulin resistance in skeletal muscle and liver (83);inhibits the insulin receptor (83); impairs insulin secretion from pancreatic β-cells (84)
SHBG	*Shbg/SHBG*	95	Decreased	Suppresses lipogenesis in liver, exacerbates steatosis (85)	Unknown
TSK	*Tsku/TSKU*	~40	Increased	Promotes steatosis and NASH (86)	Associated with whole-body insulin resistance (86)

Abbreviation: ATGL, adipose triglyceride lipase.
[a]Molecular weight is predicted and requires form validation.

maintain body temperature during cold exposure (51). Additionally, administration of recombinant activin E to mice with activin E deficiency (via global knockout of follistatin-like 3) resulted in hepatic steatosis, suggesting a role in regulating lipid metabolism (50). The expression pattern in obesity is interesting, and potentially unexpected, as on the one hand plasma activin E levels are increased in individuals with obesity, but on the other hand mice with increased circulating activin E levels are resistant to weight gain. Further studies are required to assess how activin E drives uncoupled respiration and why this mechanism does not prevent weight gain in individuals with obesity.

Adropin

Adropin is encoded by the *ENHO* gene, and expression is decreased in response to elevated hepatic lipid availability (90). Serum adropin levels are lower in humans with obesity (91) and in patients with type 2 diabetes (92), and although there is no reported link between serum adropin and NAFLD in humans, high-fat feeding reduces *ENHO* expression in parallel with the development of hepatic steatosis in mice (93). In line with these observations, hepatic steatosis is exacerbated in adropin-null mice, and this is accompanied by impaired glucose tolerance and insulin sensitivity at the whole-body level (93) and, perhaps paradoxically, increased hepatic and whole-body fatty acid oxidation (94). In agreement, hepatic steatosis resulting from high-fat feeding is attenuated in mice with adropin overexpression, and this occurs in parallel with enhanced whole-body insulin sensitivity, glucose tolerance, and lower fatty acid oxidation (52). Although little is known regarding the direct effects of adropin on metabolism, acute IP injection of adropin increases insulin signaling through protein kinase B (AKT) and AKT substrate of 160 kDa (AS160) phosphorylation, resulting in greater glucose transporter type 4 (GLUT4) translocation to the sarcolemma in skeletal muscle (53). Consistent with these effects, acute administration of recombinant adropin improves glucose homeostasis in insulin-resistant diet–induced obese mice, and it is associated with lower expression of lipogenesis-associated genes, including sterol CoA desaturase 1 and fatty acid synthase, in the liver and adipose tissue (52). Others have shown that adropin increases glucose uptake and oxidation via activation of pyruvate dehydrogenase, the rate-limiting enzyme for mitochondrial pyruvate transport, and is associated with downregulation of carnitine palmitoyl-transferase I (CPT1) activity and other proteins involved in lipid metabolism, including the fatty acid transporter CD36 (53) (Table 1). Hence, high adropin expression and secretion appear to improve insulin sensitivity and carbohydrate and lipid metabolism while suppressing hepatic steatosis. Taken together, these studies suggest that restoring adropin levels within the liver and/or blood of patients with obesity could be a therapeutic approach for NAFLD and insulin resistance.

Angiopoietin-like protein 4

Angiopoietin-like protein 4 (ANGPTL4) is secreted by liver and adipose tissue (95) and plays important roles in regulating lipid metabolism. ANGPTL4 increases

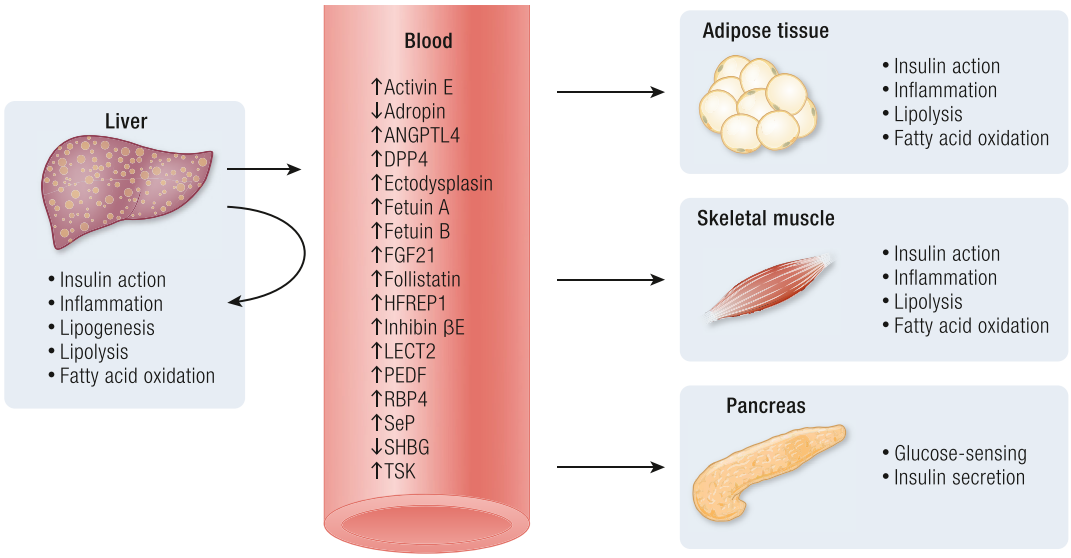


Figure 1. General metabolic processes affected by hepatokines in NAFLD. Liver secretion of various proteins are altered with NAFLD, and selected proteins can influence insulin responsiveness. In adipose and skeletal muscle, many hepatokines affect pathways involved in inflammation, lipogenesis/lipolysis, and fatty acid oxidation, which can promote insulin resistance. Additionally, some hepatokines influence insulin secretion by the pancreas, which can independently affect peripheral tissue glucose uptake and metabolism.

adipocyte lipolysis through a process that is dependent on its C-terminal fibrinogen-like domain (96), and it suppresses lipoprotein lipase activity via its N-terminal coiled-coil domain (97). This increases plasma free fatty acids and triglycerides, which is associated with ectopic lipid accumulation in liver and skeletal muscle (54, 55, 98) and is highlighted by dyslipidemia in mice with liver-specific overexpression of *Angptl4* (99). Consistent with these functions, individuals heterozygous or homozygous for the loss-of-function *Angptl4* variant E40K have significantly lower fasting plasma triglyceride levels (100) whereas *Angptl4*$^{-/-}$ mice have increased VLDL clearance (101). Free fatty acids upregulate *Angptl4* expression via peroxisome proliferator-activated receptor (PPAR)α activation in the liver and PPARγ in adipose tissue (102), demonstrating the likelihood of a feed-forward loop whereby lipid oversupply drives *Angptl4* expression and high ANGPTL4 levels further drive dyslipidemia. Such regulation would be clinically unfavorable for PPAR agonists, which are used as antidiabetic and lipid-lowering agents. Insulin suppresses hepatic *Angptl4* expression; however, this mechanism is likely to be impaired in insulin-resistant states (103).

The role of ANGPTL4 on insulin action and glycemic control is controversial. Although mice with ANGPTL4 overexpression have severe hepatic steatosis, they exhibit improvements in hepatic and systemic insulin sensitivity (55, 98). In agreement, ANGPTL4 increases insulin-mediated inhibition of gluconeogenesis and decreases hepatic glucose production in primary hepatocytes, and in humans ANGPTL4 levels correlate positively with insulin sensitivity (55). In contrast, reducing plasma ANGPTL4 via genetic deletion in mice reduces blood lipids, reduces ectopic lipid accumulation in liver and muscle, enhances insulin signaling, and improves glycemic control (57, 58, 101), and anti-ANGPTL4 antibody therapy in obese and diabetic mice recapitulates this favorable metabolic phenotype (101). Further studies are clearly required to fully understand the discrepancy in these disparate findings and to ascertain the potential of ANGPTL4 therapeutic applications in dyslipidemia and glycemic control.

Dipeptidyl peptidase-4

Dipeptidyl peptidase-4 (DPP4) is a ubiquitous serine protease secreted by the liver that rapidly inactivates the circulating incretin hormones glucagon-like peptide (GLP)-1 and gastric inhibitory peptide (GIP) (60). Incretins are important regulators of whole-body glucose homeostasis, as GLP-1 and GIP promote insulin secretion and suppress glucagon secretion, resulting in peripheral glucose uptake and reduced hepatic glucose output (60). Individuals with NAFLD and insulin resistance have elevated plasma DPP4 activity (59), which is consistent with lower GLP1 and GIP levels in the blood of these individuals (104). Consistent with human studies, liver-specific overexpression of DPP4 impairs whole-body glucose tolerance in high-fat–fed mice, effects that are linked to reduced circulating GLP-1 (59). DPP4 is likely to directly affect metabolism in peripheral tissues, as treatment of primary hepatocytes, adipocytes, and skeletal myotubes with recombinant DPP4 impairs insulin sensitivity (60, 105). However, a receptor for DPP4 has not yet been identified. DPP4 also drives liver steatosis, most likely by increasing fatty acid uptake and storage in hepatocytes (60). In agreement with these observations, genetic ablation (106) or administration of oral DPP4 inhibitors such as vildagliptin (107) or sitagliptin (108) improves both hepatic steatosis and glucose tolerance, further highlighting the systemic and autocrine/paracrine actions of DPP4.

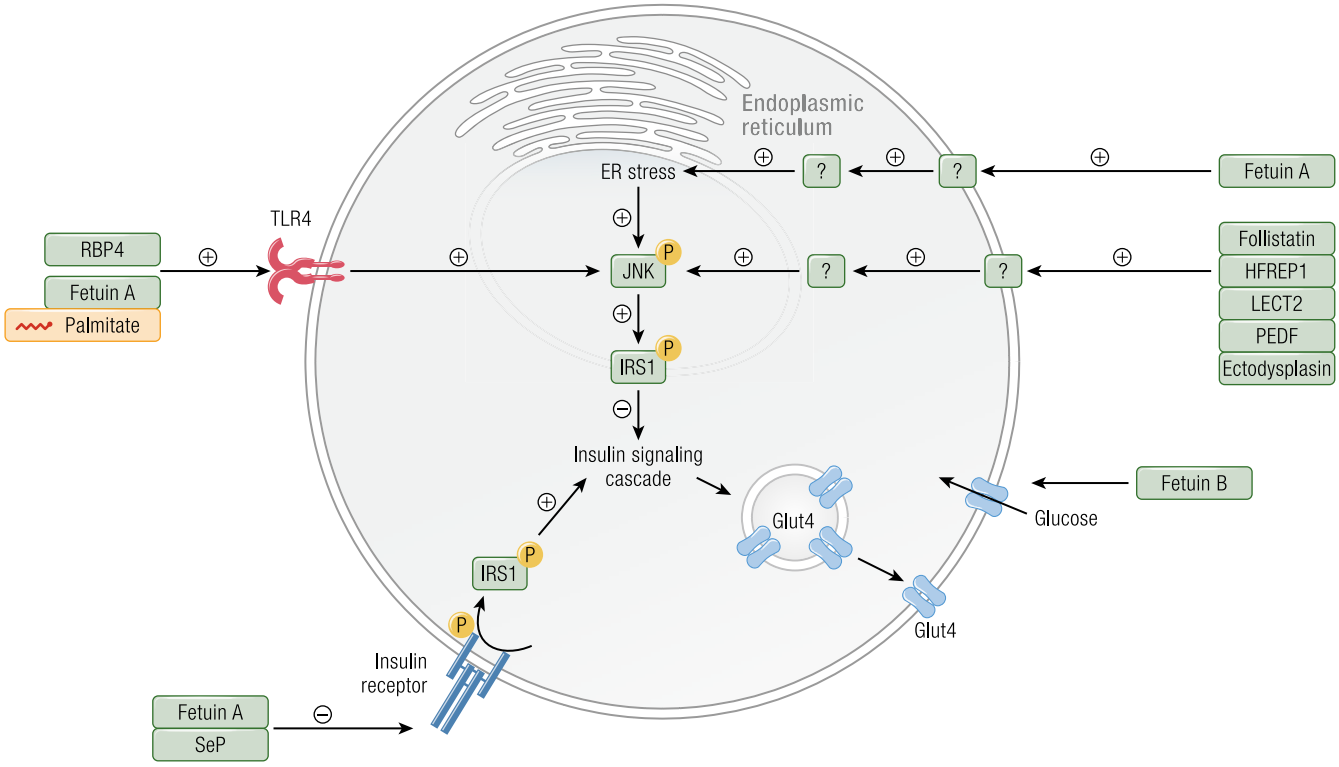
Ectodysplasin A

Ectodysplasin A is a newly discovered hepatokine that is associated with obesity and insulin resistance (61). Liver and serum ectodysplasin A levels are increased in high-fat–fed mice and mice with genetic obesity (*i.e.*, leptin receptor–deficient db/db mice), and liver ectodysplasin A mRNA levels increase with steatosis severity and correlate with a reduction in whole-body insulin action in humans (61). In parallel with these findings, treatment of C2C12 skeletal myotubes or whole-body overexpression of ectodysplasin A in mice induces muscle insulin resistance in association with activation of c-Jun N-terminal kinase (JNK), and these effects were reversed upon partial silencing of ectodysplasin A in mice (61) (Fig. 2). Notably, ectodysplasin A did not affect hepatic insulin action or other parameters of energy homeostasis, pointing to a direct liver-to-muscle crosstalk. Additional work is required to determine the role of ectodysplasin on lipid metabolism and confirm the relevance of this protein in NAFLD and type 2 diabetes.

Fetuin A

Fetuin A (also known as α-2-HS-glycoprotein) is a liver-secreted glycoprotein encoded by the *ASHG* gene. Fetuin A is positively associated with circulating triglycerides, the severity of NAFLD (109, 110), and insulin resistance (111, 112) in rodents and humans. In this regard, lipid oversupply induces ER stress, which activates extracellular signal–regulated kinase (ERK)1/2 and JNK to drive fetuin A production (62). The hepatic expression and secretion of fetuin A is also regulated by F-box and WD repeat domain-containing 7 (FBXW7), a ubiquitin protein ligase that degrades fetuin A. FBXW7 is suppressed in obese mice and in humans with obesity in parallel with increased fetuin A levels (113). Fetuin A induces insulin resistance by several mechanisms (64, 113–116) (Fig. 2). Fetuin A inhibits insulin receptor tyrosine kinase activity, resulting in lower autophosphorylation and impaired insulin signaling (63). Fetuin A also acts as an endogenous ligand for Toll-like receptor (TLR)4, which

Figure 2. The influence of hepatokines on insulin resistance in skeletal muscle or adipose tissue. Hepatokines target pathways involved in regulating insulin action. Fetuin A (in the presence of palmitate) and RBP4 can activate TLR4 and result in JNK phosphorylation, greater serine phosphorylation of IRS1, and suppression of insulin signaling and GLUT4 trafficking to the sarcolemma. Fetuin A and SeP can directly inhibit the insulin receptor, resulting in lower insulin signaling and GLUT4 trafficking. Fetuin A can promote ER stress through unknown mechanisms, resulting in greater JNK activation and impaired insulin signaling. Follistatin, HFREP1, LECT2, PEDF, and ectodysplasin are also implicated in impairing insulin signaling secondary to activation of JNK; however, the upstream signaling is unresolved.



enables saturated free fatty acids to activate TLR4 signaling to induce insulin resistance (64). This fetuin A/free fatty acid interaction predicts the development of insulin resistance in humans (117). In the pancreas, fetuin A signals to β-cells and impairs glucose sensing (118), which results in impaired insulin secretion (118, 119) in response to inflammatory processes, including TLR4, JNK, and nuclear factor κB (NF-κB) activation and the accumulation of lipotoxic lipids (119, 120). Mice with global fetuin A deletion show improved insulin sensitivity and are resistant to diet-induced obesity (115, 121), highlighting the likely therapeutic potential of fetuin A antagonists.

Fetuin B

Fetuin B is encoded by the *FETUB* gene and shares 22% homology with fetuin A. Fetuin B is increased in patients with NAFLD (65, 110, 122, 123), type 2 diabetes (123, 124), and gestational diabetes (125, 126), and it correlates positively with insulin resistance (124). Although administration of fetuin B induces insulin resistance in myotubes and hepatocytes *in vitro*, administration of fetuin B in lean mice caused whole-body glucose intolerance but not insulin resistance (65), observations that were recapitulated in humans (110). This indicates that fetuin B's primary function is the suppression of glucose effectiveness (65), which refers to the ability of glucose to promote its own disposal, independently of insulin (Fig. 2). This process is at least as important as insulin for glucose clearance, accounting for ~50% of an oral glucose tolerance test in normal individuals and ~80% in insulin-resistant individuals with obesity (127). Additionally, there is evidence that fetuin B impairs first-phase glucose-stimulated insulin secretion (124), signifying a role in β-cell function. The importance of fetuin B in NAFLD-induced insulin resistance is confirmed by studies showing that short hairpin RNA suppression of liver fetuin B protein and reduced fetuin B secretion improve glycemic control in obese, insulin-resistant mice (65).

Fibroblast growth factor 21

Fibroblast growth factor (FGF)21 regulates systemic lipid metabolism in response to diet, exercise, and cold exposure (128, 129), and its role in metabolism has been extensively reviewed (129). FGF21 expression is increased by activation of PPARα (66), and consistent with this finding, humans with NAFLD have increased circulating FGF21 levels (130, 131). This may be a

compensatory response designed to limit the impact of lipotoxic stress, as FGF21 reduces adipose tissue lipolysis (67, 132), increases fatty acid oxidation, lowers hepatic lipids such as diacylglycerol, enhances insulin sensitivity, and improves glycemic control (68, 133, 134). FGF21 also reduces hepatic VLDL secretion and accelerates VLDL disposal in both white and brown adipose tissue via coordinated upregulation of CD36 and lipoprotein lipase (135). The importance of FGF21 is highlighted in mouse studies where the deletion of *Fgf21*, its receptor FGF receptor-1c, or the coreceptor β-klotho, results in greater adiposity, hepatic steatosis, and liver insulin resistance, increased hepatic glucose production, and hyperglycemia (136). Finally, FGF21 promotes pancreatic β-cell function and insulin secretion (69). It is for these reasons that FGF21 has emerged as a therapeutic agent for the treatment of type 2 diabetes and the metabolic syndrome (137), although FGF21 analogs have failed to lower blood glucose in humans.

Follistatin

Follistatin is a member of the TGFβ family and was originally recognized for its inhibitory effect on FSH production in the pituitary (138) and, later, suppression of myostatin to support skeletal muscle growth, which demonstrated actions outside of the reproductive system (139). Follistatin is increased in serum of individuals with NAFLD (89) and type 2 diabetes (98), potentially through forkhead box O1 (FOXO1)–mediated transcriptional activation (140). In contrast, weight loss following bariatric surgery leads to a reduction in serum follistatin, which is accompanied by improvements in insulin sensitivity and glycemic control (140, 141). It remains to be determined whether the decrease in serum follistatin is a direct mediator of the improvements in glycemic control. However, it is known that follistatin promotes proinflammatory cytokine expression, such as IL-1β, that is implicated in fibrosis progression (70, 142) and the development of insulin resistance in adipose tissue (143) and skeletal muscle (144). Similarly, follistatin overexpression in isolated hepatocytes or livers of mice impairs signaling in white adipose tissue, insulin-mediated suppression of hepatic glucose production, and whole-body glucose tolerance, whereas follistatin knockdown improves insulin sensitivity (140) (Fig. 2). Taken together, these studies provide evidence that follistatin is increased with NAFLD and can promote inflammation, insulin resistance, and glucose intolerance.

Hepatocyte-derived fibrinogen-related protein 1

Hepatocyte-derived fibrinogen-related protein 1 (HFREP1), also referred to as hepassocin or fibrinogen-like protein 1, is secreted by hepatocytes and is known to promote cell growth and proliferation (145–147). More recently, HFREP1 has been implicated in NAFLD and systemic insulin resistance. HFREP1 is elevated in human NAFLD (72) and NASH (148), and circulating concentrations correlate positively with plasma glucose levels and insulin resistance (72). In mice, high-fat feeding promotes liver HFREP1 expression and NAFLD (71), suggesting that elevated circulating lipids induce hepatic HFREP1 expression. In this context, the fatty acid palmitate (C16:0) dose-dependently increases HFREP1 expression in hepatocytes (149). Lipid-dependent HFREP1 expression is mediated by ER stress and the resultant activation of the P38 and CCAAT/enhancer-binding protein β in hepatocytes, and pharmacological blockade of this pathway blunts HFREP1 expression and partially restores insulin action (149). This provides a plausible mechanism for NAFLD-induced HFREP1 production, given that ER stress is elevated in humans with conditions characterized by dyslipidemia such as NAFLD (150), diabetes (151), and obesity (152). Moreover, HFREP1 promotes lipogenesis through ERK1/2 activation (72), indicating the presence of a feed-forward mechanism that drives NAFLD. HFREP1 has also been shown to cause insulin resistance in immortalized C2C12 myotubes and skeletal muscle *ex vivo* (72), which is mediated by decreased 5′-AMP–activated protein kinase (AMPK) phosphorylation and enhanced JNK activation in a process dependent on epidermal growth factor receptor and FOXO1 phosphorylation (149). Administration of recombinant HFREP1 or genetic overexpression of HFREP1 causes liver and skeletal muscle insulin resistance (71), whereas deletion of liver HFREP1 protects against diet-induced insulin resistance in mice (72) (Fig. 2). Reducing circulating HFREP1 could therefore be a viable approach for insulin resistance.

High-mobility group box 1 protein

Sterile inflammation caused by free fatty acids, chemokines, and cytokines stimulates the release of endogenous molecules termed damage-associated molecular patterns, and these molecules can activate TLR signaling in a variety of cell types that promote inflammatory responses. High-mobility group box 1 protein (HMGB1) was originally discovered as a protein that binds to nucleosomes to stabilize DNA structure and modulate transcription (153), but more recently it has been shown to be secreted from hepatocytes (75). HMGB1 expression and secretion are increased in NAFLD, and this process is mediated by increased free fatty acid availability (75). The secreted HMGB1 activates TLR4 signaling and the resultant NF-κB activation drives inflammation in neighboring hepatocytes. Given that TLR4 activation dampens insulin signaling (114), it is possible that HMGB1–TLR4 signaling may contribute to insulin resistance in NAFLD, although this requires formal testing. Blocking HMGB1 reduces lipotoxic effects in hepatocytes (75) and protects against NAFLD progression

in rats (73, 74), which supports a prominent autocrine/paracrine role. Whether the pathogenic effects of HMGB1 extend beyond the liver in humans is questionable, as HMGB1 levels are not associated with histological severity in NAFLD (154).

Inhibin βE

The hepatokine inhibin βE is a member of the TGFβ family and is positively associated with body mass index and insulin resistance in rodents (155, 156) and humans (155). Knockdown of hepatic inhibin βE using small interfering RNA attenuates fat mass gain in parallel with greater whole-body fat oxidation in obese db/db mice (155); however, no overt metabolic phenotype was reported in *Inhbe* knockout mice (157). Additional work is needed to determine whether inhibin βE indeed increases fat oxidation and whether blocking inhibin βE's actions has therapeutic utility for NAFLD and perhaps obesity.

Leukocyte cell–derived chemotaxin 2

Leukocyte cell–derived chemotaxin 2 (LECT2) is a 16-kDa hepatokine originally described as chemotactic for neutrophils (158). LECT2 has since been implicated in NAFLD (159, 160) and insulin sensitivity (159, 160), and circulating LECT2 expression is positively correlated with body weight and insulin resistance in humans (76). Studies in mice show that whole-body deletion of *Lect2* enhances insulin-stimulated AKT phosphorylation in skeletal muscle, whereas administration of recombinant LECT2 activates JNK (76) and inhibits insulin signaling via increased serine phosphorylation of IRS1 (77) (Fig. 2). Interestingly, pharmacological inhibition of DPP4 improves glucose metabolism in mice in parallel with a reduction in hepatic LECT2 protein content, and this occurs via activation of AMPK and suppression of JNK activity (161). Thus, DPP4 may regulate LECT2 expression, indicating likely cross-talk between hepatokines in NAFLD.

Pigment epithelium-derived factor

Pigment epithelium–derived factor (PEDF) is a 50-kDa noninhibitory serine protease originally found to be secreted by retinal epithelial cells, but it is also highly expressed in, and secreted by, liver and adipose tissue (162). Liver PEDF expression (163) and circulating PEDF levels (164–166) are increased in humans with obesity, insulin resistance, and NAFLD, and it is reduced with weight loss (166). PEDF is implicated in the development of insulin resistance and glucose intolerance in mice (80). PEDF increases JNK and ERK1/2 activity in skeletal muscle and liver, as well as NF-κB activity in adipocytes, which corresponds with reduced insulin signal transduction (80, 167) (Fig. 2). Increasing plasma PEDF by administration of recombinant PEDF or overexpression of *Pedf* in adipose tissue of mice increases adipose tissue lipolysis via its interaction with adipose triglyceride lipase (ATGL), the rate-limiting enzyme for triglyceride hydrolysis, and this coincides with increased ceramide and diacylglycerol accumulation in liver and muscle (78, 80, 167, 168). Neutralizing PEDF with monoclonal antibodies reverses these effects (80). Whereas circulating PEDF correlates with hepatic steatosis and insulin resistance, liver-specific overexpression of *Pedf* reverses hepatic lipid accumulation (169), which likely reflects higher intrahepatic lipolysis, and it aligns with lower NASH development (170, 171). Taken together, this suggests that the increased production of PEDF with NAFLD is directed toward secretion and not retained within the liver, where it promotes insulin resistance and dyslipidemia.

Retinol binding protein 4

Retinol binding protein 4 (RBP4) is a liver and adipose tissue secreted protein that transports vitamin A in the form of retinol (172). Serum RBP4 levels are increased with NAFLD (173, 174), insulin resistance (175), and in type 2 diabetes (176), and reduced with diet-induced weight loss (177), bariatric surgery (178), and exercise (176). Although these findings in humans suggest a potential role for RBP4 in reducing insulin action, direct examination of RBP4 effects in mice are equivocal. RBP4 overexpression causes inflammation and insulin resistance in mouse adipose tissue due to activation of JNK and TLR4 signaling (82), and genetic deletion of *Rbp4* enhances insulin sensitivity (179) (Fig. 2). However, in a recent study where RBP4 was overexpressed specifically in the liver of mice, glycemic control was not affected despite significant increases in circulating RBP4 levels (81). In light of these conflicting data, additional research is warranted to determine RBP4's role in the pathogenesis of NAFLD and type 2 diabetes.

Selenoprotein P

Circulating selenoprotein P (SeP) levels are positively correlated with type 2 diabetes (83), insulin resistance, and blood glucose levels in humans (180–182), which agrees with studies in mice reporting increased liver expression and secretion of SeP in response to high-fat feeding (83), NAFLD (183), and type 2 diabetes (83). SeP expression and secretion are increased in response to ER stress and JNK activation, subsequent to TLR4 stimulation (184). Moreover, activation of AMPK via the anti-inflammatory drug salsalate or the insulin-sensitizing drug metformin protects against ER stress and SeP production (184). Taken together, this links proinflammatory and/or dyslipidemic states to increased SeP expression.

Consistent with a role in metabolic disease, acute administration of recombinant SeP causes insulin resistance in mice (83). *In vitro*, SeP administration impairs insulin receptor phosphorylation in HepG2 hepatocyte-like cells, suggesting direct inhibition of

"...ketone bodies signal to peripheral tissues and the central nervous system to regulate metabolism."

autophosphorylation at this proximal step of insulin signaling (83) (Fig. 2). Additionally, SeP impairs glucose-stimulated insulin secretion in pancreatic β-cells, effects that are reversed by monoclonal antibody neutralization of circulating SeP (84). Genetic deletion and RNA interference–mediated knockdown of SeP improves systemic insulin sensitivity and glucose tolerance (83), whereas SeP-neutralizing antibodies improve insulin secretion and glycemic control in diabetic mice (84), collectively highlighting the potential therapeutic utility of this hepatokine.

Sex hormone binding globulin

Sex hormone binding globulin (SHBG) is best known as a transporter of sex steroids (185, 186). Liver (187) and serum (188) SHBG levels are lower in individuals with hepatic steatosis when compared with individuals with no adverse liver pathology, and plasma SHBG negatively predicts insulin resistance and hyperinsulinemia (187–191). This is accompanied by lower hepatic SHBG content and lower expression of the transcription factor hepatocyte nuclear factor 4α, a key transcriptional regulator of SHBG (187). Additionally, low circulating SHBG is independently associated with obesity, and weight loss following bariatric surgery increases circulating SHBG levels (192). Collectively, these data describe a clear association between obesity, NAFLD, insulin resistance, and lower SHBG levels in liver and blood.

The lower expression of SHBG in NAFLD may occur secondary to inflammation, as an increase in TNFα in response to JNK and NF-κB activation reduced SHBG production in HepG2 cells (193). Given that insulin promotes SHBG production *in vitro* (194), and people with type 1 diabetes have a stronger relationship between insulin levels and SHBG than do those with type 2 diabetes (189), it is possible that hepatic insulin resistance could precede a reduction in SHBG and exacerbate lipid accumulation. This notion is at least partially supported by the finding that resveratrol, a polyphenol that improves insulin sensitivity, also increases SHBG levels in mice (195). The mechanisms underpinning the effects of SHBG on glycemic control are unknown. With respect to lipid metabolism, overexpression of SHBG suppresses lipogenesis (85, 196) that could reduce hepatic steatosis.

Tsukushi

Tsukushi (TSK) is a highly conserved proteoglycan in mammals and a newly discovered hepatokine that is increased in rodent obesity (197) and NASH (86). Studies in *Tsk*-null mice highlight an important role for TSK in regulating energy balance under obesogenic conditions. TSK is induced in response to increases in energy expenditure, which blunts sympathetic outflow and innervation of adipose tissue, thereby reducing thermogenesis and energy expenditure (197). Ablation of TSK also prevents diet-induced NASH and whole-body insulin resistance (86), which may be mediated by the improvements in systemic metabolism rather than direct actions targeting proinflammatory, profibrotic, or insulin signaling pathways. Future work including the identification of the putative TSK receptor is required to determine the role of TSK in lipid metabolism and insulin signaling in peripheral tissues.

Hepatocyte exosomes and protein secretion

Exosomes are emerging as an important mode of intercellular and intertissue communication. Proteomic analysis of exosomes derived from rat primary hepatocytes identified ~250 proteins, some of which are also denoted as classically secreted hepatokines (*e.g.*, DPP4). Bioinformatic analysis predicts that these hepatocyte-derived exosomal proteins are implicated in intracellular transport, lipid metabolism, carbohydrate metabolism, metabolite availability, and protein turnover (198). Although the influence of NAFLD or NASH on liver-secreted exosomes is poorly understood, *in vitro* studies showed that palmitate exposure increases exosome/extracellular vesicle secretion (199–201), and that these exosomes contained a greater proportion of proteins involved in regulating fibrosis (*i.e.*, α-SMA, TGFβ, Col1a1) (201). These exosomes were found to communicate with "healthy" hepatocytes to promote the progression of fibrosis (199–201), suggesting a potential autocrine effect of exosomes in liver disease. However, this is very much an emerging field, and the importance of liver-derived exosomes in regulating cell metabolism and influencing whole-body glucose homeostasis is currently unknown. Future work should examine how liver disease affects the protein composition in liver-secreted exosomes, and whether these exosomes are important mediators of insulin resistance.

Summary

Hepatokines clearly exert pleiotropic effects on lipid and glucose metabolism and insulin action, and their secretion is impacted by NAFLD (Table 1). Although there has been one comprehensive examination of the hepatocyte protein secretome (65), which demonstrates marked changes in response to simple steatosis, rapid developments in mass spectrometry now permit a deeper examination of cell/tissue secretomes, enabling more detailed understanding of the breadth of protein secretion during the progression of NAFL to NASH. Performing such experiments in human liver tissues will be important in driving our understanding of hepatokines in physiology and NAFLD-related comorbidities and, by extension, the identification of therapeutic targets to treat cardiometabolic diseases. Such studies will also be important for identifying novel biomarkers that could be used in conjunction with readily available clinical parameters to identify the presence and staging of NAFLD/NASH with higher sensitivity and specificity than current risk-stratification algorithms used in routine clinical care.

Metabolite secretion, NAFLD, and insulin resistance

The liver plays critical roles in regulating systemic glucose and lipid metabolism, processes that have been extensively reviewed in the context of NAFLD (202). Glucose and VLDL aside, liver-derived major metabolite classes, including lipoproteins, ketones, acylcarnitines, and bile acids, appear to transduce specific metabolic signals; however, much of the literature on this topic remains correlative and circumstantial with regard to their endocrine functions. This mode of intertissue communication is discussed below with this caveat in mind.

Ketones

Ketone bodies such as acetone, acetoacetate, and β-hydroxybutyrate are produced in the liver using β-oxidation–derived acetyl-CoA as substrate, and they are then secreted from the liver for transport to other peripheral tissues. Upon their uptake by peripheral tissues, ketone bodies are converted back to acetyl-CoA, which provides substrate for energy production through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. The excess acetyl-CoA also increases the capacity for protein acetylation, particularly of mitochondrial proteins, which regulates the activity of transcription factors and transcriptional coactivators such as FOXO1, PPARγ coactivator 1α (PGC1α), and PPARγ, all of which play critical roles in regulating the expression of metabolic genes, particularly in response to changes in nutrient availability (203, 204). In this regard, ketogenesis is important when carbohydrate is limiting, including starvation and with the consumption of extremely low-carbohydrate diets, which are often referred to as "ketogenic" diets (205, 206). Ketone bodies contribute up to 20% of total energy expenditure under these conditions (207, 208).

Moving beyond their direct role as an energy substrate, ketone bodies signal to peripheral tissues and the central nervous system to regulate metabolism. Ketones can cross the blood–brain barrier and are sensed in the hypothalamus to stimulate food intake by increasing the expression of the orexigenic neuropeptides *Npy* and *Agrp* (209) and through the potentiation of hypothalamic leptin and insulin signaling (210) (Fig. 3). Such signaling is associated with reduced adiposity and improved systemic insulin sensitivity (210). In the periphery, β-hydroxybutyrate regulates lipid metabolism in adipocytes via activation of hydroxy-carboxylic acid receptor 2 (HCA2, GPR109A), which sequentially decreases adenylyl cyclase activity, protein kinase A activity, and lipolysis (211, 212). β-Hydroxybutyrate is also implicated in the longer-term regulation of metabolism by modifying histones through two distinct epigenetic processes. First, β-hydroxybutyrate inhibits class I histone deacetylase (HDAC) (213), which is associated with reduced oxidative stress (213), a well-documented mediator of insulin resistance (214). Second, β-hydroxybutyrate directly modulates lysine residues on histones via a process known as lysine β-hydroxybutyrylation, leading to activation of starvation-regulated metabolic pathways, including amino acid catabolism, PPAR signaling, and oxidative phosphorylation (215). Although correlative, HDAC inhibition is reported with the consumption of ketogenic diets, with subsequent activation of PPARα and the expected sequalae of increased expression of lipid metabolism genes (216), greater hepatic fatty acid oxidation and plasma triglyceride clearance, and FGF21 production (66, 217). Thus, β-hydroxybutyrate links changes in metabolite-directed histone modifications to changes in cellular metabolism.

Although β-hydroxybutyrate is commonly used in metabolic studies to represent "ketones," acetoacetate constitutes 25% to 50% of the total hepatic ketone body pool (218, 219) and is thereby a significant source of acetyl-CoA and a potential signaling molecule. Acetoacetate can inhibit glucose uptake in skeletal muscle and heart (220, 221), although others show no effect (222). Recent work has shown that hepatocyte-secreted acetoacetate, but not β-hydroxybutyrate, ameliorates diet-induced hepatic fibrosis (223), but these studies did not assess metabolism, and a better understanding of acetoacetate roles in metabolism is needed. Similarly, another ketone, acetone, can be taken up by tissues but its effects on glycemic control and insulin sensitivity are unknown. Further detailed information on ketones and metabolism is available elsewhere (224).

The role of ketones in regulating insulin action and glycemic control is equivocal (225–228). These conflicting findings result from discrepancies in study designs, the composition of ketogenic diets, the likelihood of "positive" and "negative" responders to ketogenic dietary intervention, the duration of the ketogenic diet, and the myriad of "off-target" changes that accompany ketogenic diet consumption.

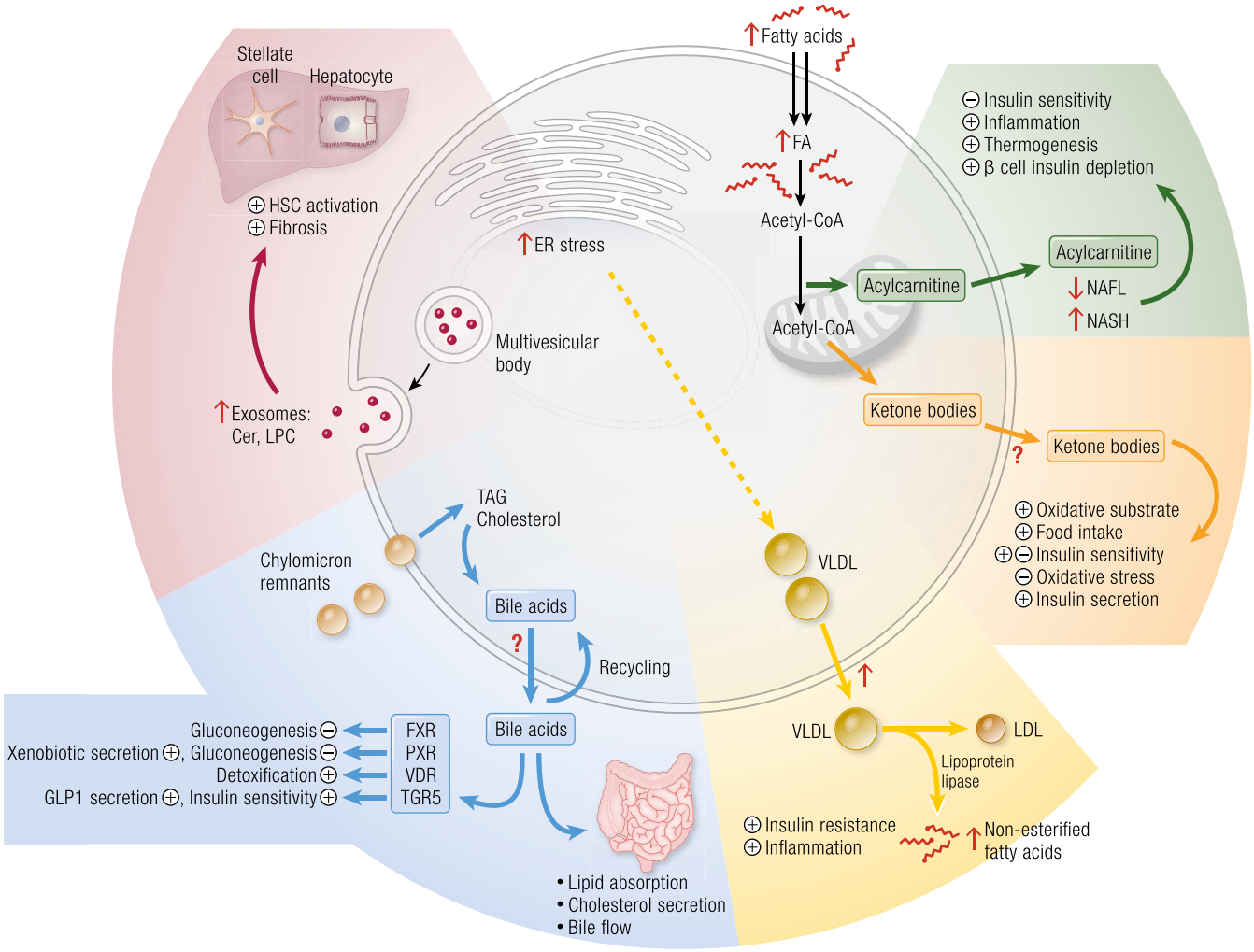
Ketone bodies in NAFLD. Hepatic ketogenesis and plasma β-hydroxybutyrate are reportedly increased (8, 229), unchanged (230–233), or decreased (234, 235) in rodents and humans with NAFLD. There is similar uncertainty in NASH, with reports of higher (236, 237) or lower levels (229) of circulating ketone bodies compared with individuals with steatosis or no liver pathology. Future studies are required to clarify this ambiguity and to also identify the potential links between specific ketones and insulin action.

Lipoproteins

The liver secretes lipids primarily within VLDLs, but also within extracellular vesicles. Although most lipids within VLDLs are triglycerides (~55%), VLDLs also contain cholesterol and cholesterol esters (~25%) and phospholipids (~20%), with most being 16:0-

"Increased circulating ceramide and/or ceramide accumulation in rodents induces inflammation and insulin resistance."

Figure 3. Hepatic metabolite secretion. Different pathways for metabolite secretion are color-coded. Fatty acids (FA) are taken up by hepatocytes and can be converted to acylcarnitine for oxidation within the mitochondria. These acylcarnitines can be secreted from the liver and induce insulin resistance in peripheral tissues (green). Ketone bodies are produced during mitochondrial fatty acid oxidation and can be secreted from hepatocytes, affecting oxidative metabolism, and they may affect insulin sensitivity in peripheral tissues (orange). The liver plays a significant role in lipoprotein metabolism by taking up chylomicron remnants from the circulation (blue) and by secreting VLDLs that transport triglycerides (and other lipids) to peripheral tissues (yellow). Cholesterol synthesized within the liver or taken up as part lipoproteins can be converted to bile acids, which are secreted and affect intestinal lipid absorption and bile flow, as well as peripheral metabolism through activation of various receptors (blue). Lastly, the liver secretes exosomes that carry thousands of metabolites, including ceramide and LPC, that were shown to lead to stellate cell activation and fibrosis in a paracrine manner (red). Changes in those pathways with NAFLD are shown with red arrows (↑ increased, ↓ decreased with NAFLD, ? unknown or controversial). HSC, hepatic stellate cell; TAG, triacylglycerol.



containing phosphatidylethanolamine (238). Additionally, 1% to 5% of liver sphingolipids, mainly ceramide and free sphingosine, can be released as components of VLDLs (239).

Fatty acids are cleaved from triglyceride contained within VLDLs by lipoprotein lipase that is localized to the surface of endothelial cells in capillaries and are transported into cells residing in close proximity or carried in the blood bound to albumin. Increasing VLDL-triacylglycerol secretion and delivery to tissues can cause peripheral insulin resistance by increasing fatty acid availability, as shown experimentally by coinfusing Intralipid (triglyceride emulsion) and heparin (lipoprotein lipase activator) (240, 241) (Fig. 3). Increased circulating VLDLs, and thereby triglycerides, is associated with hepatic steatosis, obesity, and insulin resistance (242, 243). Once depleted from triglyceride, lipoprotein particles are enriched in cholesterol and cholesterol esters to form intermediate-density lipoproteins or low-density lipoproteins (LDLs). Insulin resistance in humans is characterized by high levels of plasma cholesterol esters (244), and high circulating cholesterol

levels are associated with reduced insulin secretion, effects that can be normalized with cholesterol depletion (245). Cholesterol accumulation within the plasma membrane of skeletal muscle is associated with insulin resistance secondary to reduced GLUT4 insertion within the membrane (246, 247). These studies suggest that circulating cholesterol (liver derived or from other sources) has a negative impact on glycaemic control. We are not aware of any studies assessing the direct impact of liver-derived VLDL/LDL-associated cholesterol on glucose metabolism. However, as the liver is a major site of endogenous cholesterol synthesis and secretion (248), most peripheral effects are most likely related to liver-derived cholesterol.

Intracellular ceramide accumulation causes insulin resistance (249), and circulating ceramides appear to induce similar effects. Ceramides contained within LDLs are transferred to the plasma membrane of skeletal muscle, which leads to a reduction in insulin-stimulated GLUT4 translocation (250). LDL ceramides also induce inflammation in macrophages (250), perhaps via a TLR4-dependent mechanism, which can in turn induce insulin resistance in peripheral tissues (251). Additionally, treatment of macrophages with VLDLs increases macrophage ceramide content and promotes an M1-like macrophage polarization, leading to adipose tissue inflammation and insulin resistance in diet-induced obese mice (252).

Lipoproteins in NAFLD and insulin resistance. Hepatic steatosis is associated with increased secretion of VLDLs and increased plasma triglycerides (242, 253), and patients with NAFLD have a reduced capacity for insulin-mediated suppression of VLDL secretion (254), which may contribute to the insulin resistance and glucose intolerance that is commonly associated with NAFLD (255, 256). Furthermore, increased VLDL secretion is associated with inflammation, particularly increased TNFα production (257), and increases in TNFα can further drive hepatic VLDL secretion (258) and insulin resistance (259).

VLDL and LDL particles carry ceramide (239, 260), and hepatic ceramide secretion is increased in the presence of lipid oversupply (239, 261). Furthermore, ceramide transported in LDLs is increased in the plasma of individuals with obesity with type 2 diabetes and correlates with insulin resistance (250). The liver is the major contributor to circulating ceramide levels (262), and liver ceramide synthesis is highly dependent on fatty acid availability (261), linking dysregulated adipose tissue lipolysis to liver-derived ceramide in the circulation and NAFLD. Increased ceramide secretion in conditions of hepatic steatosis/hepatic lipid oversupply may reflect an attempt by the liver to protect itself from the deleterious consequences of intracellular ceramide accumulation (261), although there is presently no known ceramide sensing mechanism. Increased circulating ceramide and/or ceramide administration in rodents induces inflammation and insulin resistance, particularly in skeletal muscle (250, 263–265), whereas a reduction in liver and plasma ceramide after weight loss is associated with reduced inflammation and improved insulin sensitivity (262, 266, 267). Additionally, these patients showed reductions in plasma cholesterol, triglycerides, LDLs, and free fatty acids (266), increased ketone bodies and acylcarnitines, and reduced levels of branched-chain amino acids and (lyso)glycerophospholipids (267), suggesting that reductions in plasma ceramide is one of many changes that mediate systemic metabolic improvements with weight loss. Similarly, induction of hepatic ceramide degradation through increased expression of acid ceramidase within the liver and subsequent reductions in circulating ceramide are associated with improvements in hepatic steatosis and systemic insulin sensitivity (268, 269).

Acylcarnitines

Acylcarnitines are generated through coupling of acyl-CoA to carnitine for import into the mitochondrial matrix. Once inside the mitochondria, carnitine and acyl-CoA are regenerated and acyl-CoA is oxidized through β-oxidation. Acylcarnitines are an important energy source within the mitochondria, and they are also secreted into the circulation (270), either directly into the blood through the acylcarnitine transporter SLC22A1 (271) or within extracellular vesicles (272, 273). The liver (274, 275), but not skeletal muscle (275, 276), is the major source of circulating acylcarnitine and this is most pronounced with fasting (275). Acylcarnitines can provide up to 5% of the circulating carbon product from fatty acids (277) and are taken up by skeletal muscle, heart, and brown adipose tissue (278, 279). Acylcarnitine accumulation in muscle has been linked to skeletal muscle insulin resistance (280), which is thought to result as a product of a mismatch between fatty acid oxidation and TCA flux, resulting in mitochondrial stress and reactive oxygen species production (281) (Fig. 3). Some studies demonstrate increased plasma acylcarnitines in humans with insulin resistance (282, 283), and treating C2C12 myotubes with acylcarnitine modestly impairs insulin signaling (280). Whether circulating acylcarnitines impair systemic insulin action and glucose homeostasis is questionable because increasing plasma acylcarnitines via γ-butyrobetaine supplementation in mice (284) or carnitine supplementation in rats (285) does not affect glucose homeostasis. In addition to their potential role in (dys)regulating insulin action, acylcarnitines are taken up by pancreatic β-cells, leading to insulin depletion as a result of a combination of diminished insulin refill and enhanced

insulin granule release (286). Acylcarnitines activate proinflammatory signaling in macrophages (287), and liver-derived acylcarnitines are a likely fuel source for brown fat thermogenesis during cold exposure, suggesting a role for acylcarnitines in regulating energy homeostasis (279) (Fig. 3).

Acylcarnitines in NAFLD and insulin resistance. Plasma acylcarnitines have been reported to be decreased in individuals with hepatic steatosis (288) and increased in patients with NASH (289–291), while others report acylcarnitine species-specific changes with NAFLD (292). The increase in NASH patients could be attributed to the down-regulation of carnitine palmitoyltransferase 2 (CPT2) and a subsequent decrease in mitochondrial fatty acid entry and oxidation (291). These reported differences may relate to changes in mitochondrial β-oxidation with NAFLD progression (37), as β-oxidation is reduced in livers with steatosis/NAFL (293, 294) and increased in NASH (237, 295). The mechanisms underpinning NAFLD stage-specific differences in lipid metabolism remain unresolved, and it would be interesting to assess how and why β-oxidation (and subsequently acylcarnitine secretion) is altered with the progression of NAFL to NASH. At this point, it is difficult to reconcile any clear association between NAFLD, acylcarnitines, and impaired glycemic control.

Bile acids

Oxidation of cholesterol within the liver results in the generation of bile acids, including cholic acid, chenodeoxycholic acid, and deoxycholic acid. In addition to well-documented roles in intestinal lipid absorption and cholesterol metabolism (296), bile acids act as signaling molecules by activating the nuclear receptors farnesoid X receptor, pregnane X receptor, and vitamin D receptor, as well as the G-protein–coupled receptor TGR5, which are expressed within and external to the enterohepatic system (297). For the most part, bile acids induce favorable metabolic outcomes such as reduced hepatic gluconeogenesis and improved glucose homeostasis through farnesoid X receptor (298, 299) and pregnane X receptor activation (300), as well as increased energy expenditure in brown adipose tissue, and improved glucose metabolism and insulin sensitivity through TGR5 (296) (Fig. 3). Notably, these metabolic effects are unlikely to be mediated via direct actions in muscle and adipose tissue because of the very low expression of these nuclear receptors in these tissues. Readers should refer to de Aguiar Vallim *et al.* (296) for an expansive review of bile acid effects on metabolism.

Bile acids in NAFLD and insulin resistance. Detailed studies on the interaction between bile acids in the enterohepatic system and NAFLD are limited, and the small number of investigations in this area are contradicting. Most studies report increases in liver and circulating bile acids with progressive NAFLD (301–307), and circulating bile acids appear to be related to the metabolic phenotype associated with NAFLD/NASH, especially insulin resistance (308). Further work is clearly needed to bridge the gap between the understanding of bile acid secretion and their metabolic functions and influence on metabolic diseases when considering the development of targeted therapies in NAFLD.

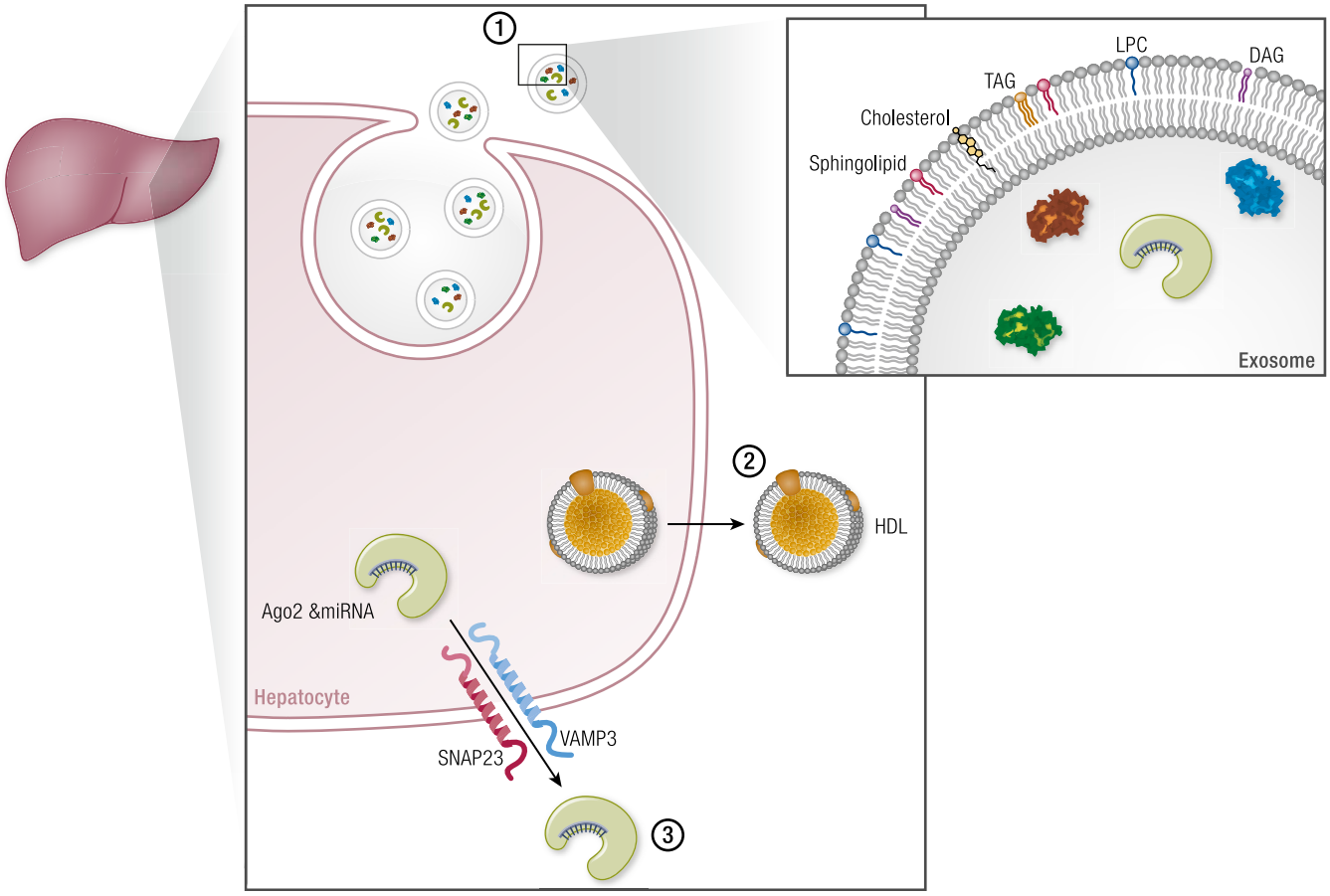
Metabolite secretion in exosomes

Most hepatic lipids are secreted within lipoprotein particles, and far less is known about the secretion of hepatic lipids or other metabolites contained within extracellular vesicles, including exosomes. In humans, ~97% of total lipids found in serum are contained within lipoproteins (309), suggesting that only a minor component of circulating lipids (~3%) are transported in extracellular vesicles (Fig. 4). This relative low abundance of lipids does not exclude a meaningful contribution of exosomal lipids as signaling molecules because exosomes can be targeted to specific cell/tissue types (310). Liquid chromatography–tandem mass spectrometry and other biochemical approaches have identified many lipid species in exosomes [*e.g.*, 280 lipid species from 18 lipid classes (311), 2000 lipid species from 22 lipid classes (273)], reflecting a complex lipid composition. The most abundant lipid classes found within exosomes are cholesterol, phospholipids, sphingolipids, and the mitochondrial lipid, cardiolipin (311–313). Although the lipid composition of exosomes generally resembles the lipid composition of the cell of origin, more so than the exosomal protein content (273, 314), certain lipids have been shown to be enriched within all exosomes, as discussed below.

Little is known about the composition of liver-derived exosomes, especially in NAFLD. Hepatocytes secrete exosomes that carry ceramides and preferentially fuse in a paracrine manner with hepatocytes (315), and one study examined the composition of immortalized Huh7 hepatocellular carcinoma cell exosomes and showed that they are enriched in long-chain saturated free fatty acids (C16:0 and C18:0), distearoyl phosphatidic acid [PA(18:0/18:0)], various phospholipids [particularly the phosphatidyl serine PS(16:1/16:1)], lysophospholipids [*e.g.*, lysophosphatidylcholines (LPCs), lysophosphatidylserines, lysophosphatidylglycerol, lysophosphatidylinositol, lysophosphatidylethanolamine), as well as a variety of sphingomyelin and cardiolipin species (273). Notably, the composition of human liver-derived exosomes and the role of the vast majority of exosomal lipids in intercellular communication are unknown.

One of the more abundant lipids within hepatocyte-derived exosomes is LPC (273). The high LPC

Figure 4. Secretion of miRNA into the circulation. Secretion of miRNA encapsulated by (1) exosomes, (2) contained within HDL, or (3) bound to argonaute 2 (Ago2), which is channeled through vesicle-associated membrane protein 3 (VAMP3) and synaptosomal-associated protein 23 (SNAP23). DAG, diacylglycerol; LPC, lysophosphatidylcholine; TAG, triacylglycerol.



content within exosomes is not surprising, as LPC is the most abundantly secreted phospholipid from the liver (316). In addition to being found within exosomes, LPC is transported in the circulation bound to albumin and is the second most prevalent phospholipid in plasma (317). Circulating LPCs activate GRP119 to enhance glucose-stimulated insulin secretion (318) and to increase glucose uptake by increasing GLUT4 translocation (319, 320), resulting in lower blood glucose levels in mice with type 2 diabetes (320). In humans, plasma LPC (and lysophosphatidylcholine/alkyl-phosphatidylcholine) levels are reduced in individuals with insulin resistance, independent of obesity (244), indicating that LPC secretion may be increased to protect against the insulin resistance associated with NAFLD. Irrespective, it remains uncertain as to how LPCs contained within exosomes or other extracellular vesicles can be transferred in meaningful quantities to impact cell functions *in vivo*.

The composition of nonlipid metabolites contained within liver-derived exosomes is not described. Metabolomic examination in other cells report high abundance of amino acids and TCA intermediates in exosomes, including acetate, citrate, pyruvate, α-ketoglutarate, fumarate, and malate, and these can used as substrates by surrounding cells (321–324).

Exosomes in NAFLD and insulin resistance. Hepatic exosome secretion is increased with NAFLD or lipid overload *in vitro* (199, 200, 325, 326). The increase in exosome release with lipid overload is mediated by ER stress (327), particularly through IRE1/XBP1 signaling (200). These lipid "overloaded" hepatocytes are enriched in C16:0 ceramide, and they are packaged into exosomes in either an IRE1/XBP1-dependent manner through ER stress-induced upregulation of the sphingolipid biosynthesis enzyme serine C-palmitoyltransferase (SPT1) (200), or through the activity of StAR-related lipid transfer domain 11 (STARD11), a ceramide transport protein (327). Increased exosomal C16:0 ceramide is also reported in mice and humans with NASH (200); however, direct secretion from the liver was not assessed in this study, and the exosome source was not verified. Ceramides

stimulate exosome secretion (328–331), and hepatocyte-derived exosomes preferentially accumulate in stellate cells and hepatocytes, where they can promote stellate cell activation and exacerbate liver fibrosis (201, 332, 333). This raises the intriguing possibility that exosomal ceramides promote NALFD progression to NASH through paracrine interactions (334, 335).

LPCs are present in hepatocyte-derived exosomes (273), but changes with NAFLD are currently unknown. Patients with NAFLD show reductions in a variety of serum LPC and phosphatidylcholine species (336–339), which is related to increased gene expression of proteins involved in LPC degradation (338). Interestingly, greater abundance of plasma LPC-16:0 can distinguish insulin-sensitive from insulin-resistant NAFL patients, providing potential diagnostic value and further supporting an insulin sensitizing role for LPCs (340).

Summary

Many studies in mice and humans have demonstrated that several liver-derived lipids and metabolites can serve as signaling molecules to regulate insulin action and other metabolic processes. Recent advances in metabolomic technologies have progressed the field by confirming correlational relationships in large human cohorts and for the identification of novel metabolites with relationships to metabolic diseases. However, the causality of most of these metabolites for the regulation of cell functions remains to be elucidated. This has been hindered by the inability to deliver lipids in aqueous environments (*e.g.*, blood, culture medium) in a reproducible, physiologically relevant manner, and new technologies are urgently required to move this field forward.

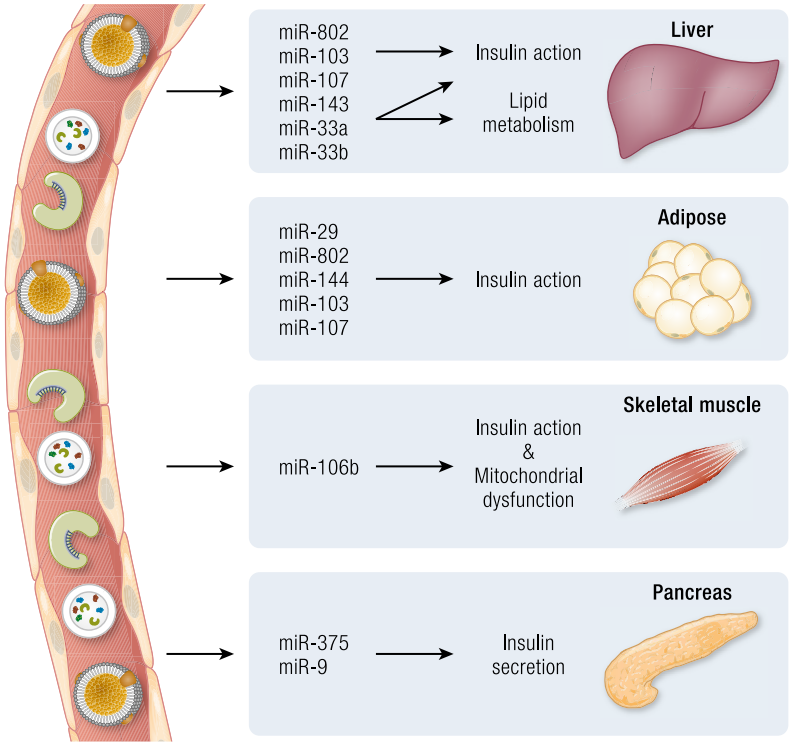


Figure 5. Potential roles of miRNA in regulating metabolism in peripheral tissues. miRNAs can be transported in the circulation and delivered to other tissues to influence metabolic functions.

miRNA secretion

miRNAs and intracellular metabolism

miRNAs are noncoding RNAs of ~22 nucleotides that regulate gene expression by binding to the 3′ untranslated region of mRNAs to repress translation or guide mRNAs for degradation in lysosomes (341). miRNAs typically target mRNAs transcribed from gene clusters rather than single genes, which facilitates critical roles in fundamental biological processes, including cell proliferation, differentiation, and apoptosis. Intracellular accumulation of specific miRNAs can regulate diverse metabolic functions in a variety of tissues, including skeletal muscle insulin action, insulin secretion from pancreatic β-cells, and adipocyte lipolysis (342, 343), thereby implicating miRNAs in the pathophysiology of metabolic diseases such as type 2 diabetes. One specific example is miR-33a and miR-33b, which are increased in NAFLD and target *IRS2* and mRNAs encoding enzymes essential for lipid transport and β-oxidation, such as CPT1a and AMPK α subunit, to simultaneously inhibit insulin signaling and lipid-supported ATP synthesis in the liver (344).

Regulation of miRNA secretion

It is now clear that miRNAs are secreted by cells and can be delivered to recipient cells where they function as endogenous miRNAs. Although miRNAs are rapidly degraded by ribonucleases in the plasma (345), miRNAs encapsulated by extracellular vesicles (346) are highly stable in the circulation (345). Moreover, miRNAs can also travel through the blood in association with proteins such as argonaute (Ago), a key element of the RNA-induced silencing complex that suppresses gene expression, and within high-density lipoproteins (HDLs) (347, 348). The Ago–miRNA complex is secreted by the interaction of vesicle-associated membrane protein 3 (VAMP3) and synaptosomal-associated protein 23 (SNAP23) to create a pore allowing export of the complex (Fig. 4).

Although a detailed discussion of the factors regulating miRNA secretion is beyond the scope of this review, there is evidence that neutral sphingomyelinase 2 (nSMase2), an enzyme that catalyzes the synthesis of ceramides from sphingomyelin, and ceramide accumulation are important for exosome secretion and miRNA sorting within vesicles (328, 349). Moreover, nSMase2 can inhibit the export of miRNA by HDLs (347), suggesting an important role for the nSMase–ceramide axis in regulating miRNA secretion. This has

important implications for miRNA "crosstalk" in NAFLD/NASH, which is characterized by increased nSMase activity and ceramide accumulation (334).

Liver-derived miRNAs and metabolic regulation

To our knowledge, no study has reported changes in miRNA secretion from the liver/hepatocytes with NAFLD. However, it is clear that many miRNAs are increased in the liver with NAFLD, that many of these miRNAs are also increased in the circulation, and that these same miRNAs can regulate insulin secretion and insulin sensitivity in various cell types (350–368) (Fig. 5). For example, miR-375 (369), miR-9 (370), and miR-143 (371) impair insulin secretion in pancreatic β-cells, and miR-29 (372, 373), miR-34a (374), miR-103 (375), miR-106b (376), miR-802 (367), and miR-144 (368) impair insulin sensitivity in the liver, skeletal muscle, or adipose tissue. Although these data suggest a potentially important role for liver-derived miRNAs in regulating glycemic control in NAFLD, definitive evidence for causality is missing, as these miRNAs are also expressed by, and secreted from, other tissues. Approaches aimed at tracking the destination cells of adipose-produced miRNAs have been developed (377), and the use of this technology would help to clarify the capacity for liver-derived miRNAs to regulate gene expression and metabolism in distant tissues. miRNAs constitute a minor fraction of all noncoding RNAs and other regulatory nucleic acids such as P-element–induced wimpy testis (PIWI)-interacting RNAs and long noncoding RNA have the capacity to regulate gene expression and to regulate glycaemic control and lipid homeostasis (378–380), and they have been detected in exosomes. Given their relatively recent discovery, it is not surprising that the relevance of these noncoding RNAs for the physiological and pathophysiological regulation of metabolism remains to be elucidated.

Conclusion

NAFLD is the most common chronic liver disorder in developed nations, and the notion that NAFLD is closely linked to the development of insulin resistance and type 2 diabetes is well accepted. Work during the last 20 years has identified a number of hepatokines that play critical roles in regulating lipid metabolism and insulin action, both in the liver and in distant tissues. The discovery of new hepatokines and an understanding of their biological functions have provided new targets for intervention strategies to stop the rise of metabolic disease, with FGF21 analogs being a prominent example.

However, we are only beginning to appreciate the sheer magnitude of factors secreted by the liver and how these are altered in NAFLD. Rapid developments in mass spectrometry have allowed for a deeper understanding of cell/tissue secretomes, and the depth of information is certain to grow. In this review, we have summarized the metabolic effects of individual hepatokines, and although this knowledge is beneficial in advancing the understanding of metabolism, both in health and NAFLD, these discoveries are tempered by the reality that a diverse array of changes link NAFLD to insulin resistance. In this context, transomic approaches using tissues obtained from well-characterized, clinically relevant human cohorts will need to be incorporated using systems biology approaches to interrogate the complex control underpinning changes in liver-secreted products and their relationship with metabolic diseases (381).

A central problem with NAFLD is that it rarely manifests specific symptoms and diagnosis is frequently incidental. Currently, the only reliable means of diagnosing and staging NAFLD is by liver biopsy, which is unsuitable for routine use on individuals at risk for NAFLD. Hence, noninvasive tests are increasingly being used in clinical practice to assess NAFLD, leading to concerted efforts to identify new serum biomarkers. Aside from informing on basic biology, future studies that interrogate the hepatocyte or liver secretomes using "omics" approaches are well positioned to identify new biomarkers for the development of readily available serum tests able to differentiate the clinically significant forms of NAFLD and to monitor disease progression noninvasively.

In closing, the field of "hepatokines" biology is rapidly evolving, and it is our hope that future work in this domain will help unravel the complexities associated with hepatokine regulation of insulin resistance in NAFLD, to delineate previously unappreciated communication between the liver and other organs in metabolic control, and advance the clinical management for the treatment of NAFLD-related comorbidities.

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Correspondence and Reprint Requests: Matthew J. Watt, PhD, Department of Physiology, University of Melbourne, Medical Building, Gratton Street, Melbourne, Victoria 3010, Australia. E: matt.watt@unimelb.edu.au.

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Abbreviations

AKT, protein kinase B; AMPK, 5′-AMP–activated protein kinase; ANGPTL4, angiopoietin-like protein 4; CoA, coenzyme A; CPT, carnitine palmytoyl transferase; DPP4, dipeptidyl peptidase-4; ER, endoplasmic reticulum; FGF, fibroblast growth factor; FOXO1, forkhead box O1; GIP, gastric inhibitory peptide; GLP, glucagon-like peptide; GLUT4, glucose transporter type 4; HDL, high-density lipoprotein; HFREP1, hepatocyte-derived fibrinogen-related protein 1; HMGB1, high-mobility group box 1 protein; JNK, c-Jun N-terminal kinase; LECT2, leukocyte cell–derived chemotaxin 2; LDL, low-density lipoprotein; LECT2, leukocyte cell–derived chemotaxin 2; LPC, lysophosphatidylcholine; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NF-κB, nuclear factor κB; nSMase2, neutral sphingomyelinase 2; PEDF, pigment epithelium–derived factor; PPAR, peroxisome proliferator-activated receptor; RBP4, retinol binding protein 4; SeP, selenoprotein P; TCA, tricarboxylic acid; TLR, Toll-like receptor; TSK, Tsukushi.