Fatty liver as a consequence and cause of insulin resistance: Lessons from type 2 diabetic liver

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Abstract. Obesity is less common in the Asian population, but Asian people may be susceptible to obesity-associated metabolic dysregulation. Accumulating evidence suggests that insulin resistance is closely associated with ectopic fat accumulation in the liver. Whether this correlation is due to a causal relationship between the conditions has long been the subject of debate. Insulin resistance and type 2 diabetes affects liver pathology, typically leading to nonalcoholic fatty liver disease (NAFLD) by dynamically altering the hepatic genes involved in glucose and lipid metabolism. Conversely, how overnutrition induces hepatic insulin resistance has been studied intensively, and has been shown to involve excessive energy flux into mitochondria, toxic lipids, reactive oxygen species, and hepatokines. In this review, we focus on NAFLD both as a consequence and as a cause of insulin resistance through lessons learned from the liver of patients with type 2 diabetes.

Key words: Fatty liver, Mitochondria, Reactive oxygen species, Hepatokine, Selenoprotein P

TYPE 2 DIABETES is a multifactorial disease that involves a genetic predisposition and/or environmental factors leading to absolute and relative deficiencies in the actions of insulin. Recent studies have unraveled the humoral and nutritional factors, and a neuronal pathway that may govern inter-organ networks [1]. The disruption of inter-organ networks leads to insulin resistance, an underlying feature involved in the pathogenesis of type 2 diabetes and its related vascular complications. Specifically, the liver functions as a control tower to maintain human body energy homeostasis by sensing nutrient stimuli via portal vein and by producing a variety of nutrients and bioactive substances. Indeed, disruption of hepatic insulin signaling in liver-specific insulin receptor knockout (LIRKO) mice results in fasting and postprandial hyperglycemia and the subsequent development of peripheral (muscle) insulin resistance [2], whereas glucose homeostasis remains normal in mice of disrupted insulin signaling both in the skeletal muscle and adipose tissue [3]. These observations suggest that hepatic insulin resistance is the primary event leading to diabetes and the subsequent development of peripheral tissue insulin resistance.

Over-nutrition is one of the major environmental factors that disrupt the inter-organ networks [1]. However, obesity is less common in Asian population. Nevertheless, Asian people may be susceptible to obesity-associated metabolic dysregulation. Accumulating evidence suggests that diabetes, obesity, and insulin resistance are closely associated with ectopic fat accumulation especially in the liver. Hepatic steatosis is often accompanied by hepatic insulin resistance. Whether this correlation is due to a causal relationship between the conditions has been the subject of considerable debate [4].

In this review, we focus on nonalcoholic fatty liver disease (NAFLD) both as a consequence and as a cause of insulin resistance mainly through lessons learned from type 2 diabetic liver. The molecular mechanisms underlying overnutrition-induced insulin resistance will also be discussed.
1. Pathology of fatty liver disease and insulin resistance

Obesity, when defined as body mass index (BMI) over 30, is less common in Asian population (Organisation for Economic Cooperation and Development (OECD) Health Data, 2008). However, cardiovascular risks and liver enzyme levels gradually increase with the development of obesity and significantly steps-up at normal BMI, suggesting that Japanese may be susceptible to obesity-associated metabolic dysregulation [5, 6]. It was recently reported that NAFLD is related to insulin resistance and cardiovascular risk factors, including diabetes, hypertension, hyperlipidaemia, and metabolic syndrome [7]. In Western people with a high prevalence of obesity, the impact of obesity is so strong on both NAFLD and metabolic abnormalities that it would be difficult to evaluate whether NAFLD itself increases insulin resistance beyond obesity. In this regard, data from lean NAFLD patients in Asia would provide insight into this question. We characterized the liver biopsy samples from patients with NAFLD and found that steatosis, but not the inflammation or fibrosis, of the liver is significantly and independently associated with homeostasis model assessment of insulin resistance (HOMA-IR) by multiple regression analysis adjusting for age, sex, BMI, and histological scores each other [8] (Table 1). Also, in the Westerners, intrahepatic fat measured by using proton magnetic resonance spectrometry, but not visceral fat quantified by magnetic resonance imaging, is associated with insulin resistance in the liver, skeletal muscle and adipose tissue evaluated by using heperinsulinemic euglycemic clamp technique with tracers [9]. Therefore, fatty liver may be an independent predictor of insulin resistance both in obese and nonobese people. This observation led us to propose a theme that addresses fatty liver as a consequence and a cause of insulin resistance.

2. NAFLD as a consequence of insulin resistance

2.1. Impact of insulin resistance on chronic liver diseases

To evaluate the impact of insulin resistance on liver pathology, we retrospectively observed a histological course of chronic hepatitis C. First, we examined the host factors associated with the progression of hepatitis C in patients with a posttransfusion hepatitis C (PTH) and found that diabetes had a great impact on the long-term prognosis of chronic hepatitis C by lowering cirrhosis-free survival rates and by reducing the time from PTH to the occurrence of hepatocellular carcinoma (HCC) and to liver-related death [10]. Coexistence of obesity with diabetes had a synergistic effect on liver fibrosis progression in patients with chronic hepatitis C [10]. Second, we analyzed the relationship of postoperative recurrence rate of HCC and coexistence of diabetes in the patients with viral hepatitis [11]. Coexistence of diabetes and insulin therapy were significant factors contributing to HCC recurrence after treatment in patients with chronic hepatitis C, but not in those with chronic hepatitis B [11]. These findings suggest that diabetes, notably insulin resistance, accelerates pathological progression of liver diseases such as hepatitis C.

NAFLD is closely associated with obesity. An adi-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Age-, sex-, and BMI-adjusted association between insulin resistance and histological changes of the liver</th>
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</thead>
<tbody>
<tr>
<td>HOMA-IR (n=131)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coefficient</td>
</tr>
<tr>
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</tr>
<tr>
<td>Grade*</td>
<td>0.67</td>
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</table>

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HOMA-IR, homeostasis model assessment of insulin resistance; All models are adjusted for age, sex, and BMI by multiple linear regression. *Three histological scores are included in the same model.
pocyte-derived hormone, adiponectin, may play a role in the pathophysiology of NAFLD through insulin-sensitizing, AMPK activating and antifibrotic effects [12]. The serum adiponectin levels are significantly lower in patients with NAFLD than in those with a normal liver, in a BMI-dependent manner. In addition, hepatic expression of adiponectin receptor AdipoR2, but not AdipoR1, is downregulated in patients with NAFLD compared with those with a normal liver, independently of BMI [13]. In a nonmalignant human hepatocyte cell line, the THLE-5b cells, fatty acids downregulates, and a thiazolidinedione, pioglitazone, upregulates mRNA and protein of AdipoR2 [13]. These findings suggest that downregulation of AdipoR2 in the liver caused by steatosis, together with decreased serum levels of adiponectin, may play a role in the development of NAFLD.

2.2. Experimental animal model of NAFLD/NASH with insulin resistance

NAFLD, particularly nonalcoholic steatohepatitis (NASH), is a global issue that will determine the future development of liver cirrhosis, liver failure, and hepatocellular carcinoma [14]. To clarify the role of insulin resistance in the development of NASH, we established the rodent models of NASH by inducing genetic and/or acquired insulin resistance in the methionine and choline-deficient (MCD) diet-induced steatohepatitis [15]. By using this NASH model with insulin resistance, we proved experimentally that 1) steatosis precedes inflammation, which leads to fibrosis in the development of MCD diet-induced steatohepatitis; 2) the pathology of steatohepatitis is more progressive in obese type 2 diabetic model OLETF rats compared with control LETO rats (Fig. 1); 3) the hepatic gene expression for transforming growth factor-β (TGF-β), alphal procollagen and plasminogen activator inhibitor-1 (PAI-1) is upregulated in OLETF rats compared with LETO rats; 3) high fat diet (HFD) further enhances insulin resistance and accelerates development of pre-cirrhosis in OLETF rats by increasing the triglyceride pool, activating stellate cells, and upregulating gene expression for sterol regulatory element-binding protein-1c and fatty acid synthase in the liver; 4) a thiazolidinedione pioglitazone attenuates the MCD diet-induced steatohepatitis in OLETF rats but not in LETO rats by reversing the underlying pathogenesis involved in this model through improvement of insulin resistance (Fig. 1).

<table>
<thead>
<tr>
<th>MCD</th>
<th>LETO rats</th>
<th>OLETF rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+High fat diet</td>
<td>+Pioglitazone</td>
</tr>
</tbody>
</table>

Fig. 1 Insulin resistance and obese diabetes accelerate the pathology in an animal model of nonalcoholic fatty liver disease

Representative photomicrographs showing the effect of a methionine and choline deficient diet (MCD), MCD+high-fat diet (+High fat diet), and MCD with pioglitazone treatment (+Pioglitazone) on liver histology in Otsuka Long-Evans Tokushima Fatty (OLETF) and Long-Evans Tokushima Otsuka (LETO) rats at 8 weeks. Hepatocyte ballooning is shown in insets. Arrows indicate infiltration of the α-smooth muscle actin (α-SMA)-positive stellate cells.

1). These results confirm a causal role of insulin resistance in the development/progression of steatohepatitis, at least partly via upregulation of genes involved in lipogenesis, inflammation, and fibrogenesis, in animal models. We also screened potential agents such as pioglitazone [15], angiotensin type 1 receptor blocker [16] and tranilast [17] that ameliorate the pathology of NASH by their unique pleiotropic mechanisms. These findings also suggest that insulin resistance is a therapeutic target of NASH.

Sanyal et al. [18] showed that vitamin E and pioglitazone ameliorate hepatic steatosis and inflammation in non-diabetic patients with nonalcoholic steatohepatitis (NASH). However, neither treatment improved hepatic fibrosis, a key feature associated with the progression of cirrhosis and hepatocellular carcinoma. Although the treatment might have been too short to reverse liver fibrosis, these negative findings suggest that the effects of pioglitazone are only marginal in non-diabetic patients. These findings seem to be compatible with the previous reports that beneficial effects of pioglitazone on liver pathology in NASH were marginal in patients without diabetes [19] compared with patients with diabetes [20], and also with our above described animal data that pioglitazone attenuates the steatohepatitis model with insulin resistance but not that without insulin resistance [15], suggesting that diabetes is an added risk for NASH.

2.3. Lessons learned from serial liver biopsies in NAFLD

In a prospective evaluation of the histological course of liver pathology, we examined whether metabolic abnormalities were responsible for the histological changes observed in 39 Japanese patients with NAFLD who had undergone serial liver biopsies [21]. The median follow-up time was 2.4 years (range, 1.0-8.5 years). Liver fibrosis improved in 12 patients (30.8%), progressed in 11 patients (28.2%), and remained unchanged in 16 patients (41%). In a Cox proportional hazard model, a decrease in A1C and bolus-first insulin therapy [22] were associated with an improvement in liver fibrosis independent of age, gender, and BMI. However, ΔA1C was more strongly associated with the improvement in liver fibrosis than was insulin use after adjusting for each factor (chi-square; 7.97 vs. 4.58, respectively) [21]. These findings suggest that tight glycaemic control, rather than weight reduction, may prevent histological progression in Japanese patients with NAFLD. In this regard, prior to the first report naming NASH by Ludwig et al. in 1980 [23], Ito et al. [24] in 1979 reported five Japanese nonalcoholic diabetic women histologically verified as having micronodular cirrhosis and named this syndrome as ‘nonalcoholic diabetic cirrhosis.’ Indeed, hyperglycemia itself may exacerbate liver fibrosis by inducing two regulators of fibrosis, TGF-β and PAI-1, both of which are upregulated in the livers of type 2 diabetic patients [25]. These findings suggest that a diabetic state itself increases the risk for liver fibrosis. Hence, glucose-lowering therapy might be a first step to prevent or reverse hepatic fibrosis in diabetic patients with NASH, whereas additional anti-fibrogenic therapy is required in non-diabetic patients.

In addition to the above described environmental factors, some genetic predispositions may be involved in NASH because only a portion of NAFLD patients (10-30%) develops NASH. A recent genome-wide association analysis of NAFLD in Japanese demonstrated that the PNPLA3 gene is strongly associated with the progression of NASH in Japanese population [26]. Such an approach will help to predict high risk NAFLD patients for developing NASH and make personalized medicine possible to select patients requiring liver biopsy and intervention.

3. NAFLD as a cause of insulin resistance

3.1. Oxidative stress that precedes the onset of insulin resistance

The liver produces and is exposed to various kind of lipids, such as fatty acids, cholesterol and triglycerides via the portal vein from diet and visceral adipose tissues. The liver and adipose tissue jointly participate in maintaining glucose and lipid homeostasis through the secretion of various humoral factors and/or neural networks. Perturbations in the inter-tissue communications may be involved in the development of insulin resistance and obesity. Visceral adiposity in obesity causes excessive free fatty acids (FFAs) flux into the liver via the portal vein and may cause fatty liver disease and hepatic insulin resistance. However, the initial events triggering the development of insulin resistance and its causal relations with dysregulation of glucose and fatty acids metabolism remain unclear. We investigated biological pathways that have the potential to induce insulin resistance by analyzing gene expression profiles in the liver and adipose tissue of mice fed an HFD before the onset of obesity and insulin resistance [27]. In the early stage during the development of an
HFD-induced insulin resistance, the pathways for reactive oxygen species (ROS) production and oxidative stress are coordinately upregulated in both the liver and adipose tissue. However, HFD is suggested to increase ROS production through discrete mechanisms because there were compensatory alterations in the expression of genes that regulate fatty acid metabolism between the liver and adipose tissue. In the liver, HFD upregulated genes involved in sterol regulatory element binding protein 1c (SREBP-1c)-related fatty acid synthesis and peroxisome proliferator-activated receptor (PPAR) α-related fatty acid oxidation. On the other hand, in the adipose tissue, HFD downregulated genes involved in fatty acid synthesis and upregulated those in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. Furthermore, increased ROS production preceded the elevation of tumor necrosis factor-α and FFAs in the plasma and liver [27]. These observations suggest that ROS may be an initial key event triggering HFD-induced insulin resistance.

Accumulating evidence has shown that insulin resistance is caused by adipose tissue inflammation. Recently, we found that CCR5, a C-C chemokine receptor, and its ligands were upregulated in the adipose tissue of mice models of obesity [28]. CCR5(-/-) mice were protected from insulin resistance, glucose intolerance, and hepatic steatosis induced by high fat feeding in relation to reduction of total adipose tissue macrophage (ATM) content and an M2-dominant shift in ATM polarization. In addition, transplantation of Ccr5(-/-) bone marrow is sufficient to protect against impaired glucose tolerance in obese mice. These findings suggest that CCR5 plays a critical role in ATM recruitment and polarization and subsequent development of insulin resistance [28]. Considering the analogy between the adipose tissue and liver in respect to infiltrating macrophages/Kupffer cells, chemokines and chemokine receptors might also play an important role during the development of hepatic inflammation/NASH and subsequent insulin resistance.

3.2. Beyond triglyceride: What is a toxic lipid?

Recent studies suggest that triglyceride itself is not a toxic lipid. Rather, the accumulation of triglycerides may be a protective mechanism to prevent toxic effects of FFAs because 1) unsaturated fatty acids rescue palmitate-induced apoptosis by channeling palmitate into triglyceride pools [29]; 2) antisense oligonucleotide-mediated inhibition of diacylglycerol acyltransferase 2 (DGAT2), that catalyzes the final step in hepatocyte triglyceride biosynthesis, decreased hepatic steatosis, but increased lobular necroinflammation, and fibrosis together with increased hepatic FFAs and lipid peroxidation/oxidant stress in db/db mice fed an MCD diet [30]. In addition, triglyceride accumulation itself does not cause insulin resistance because 1) suppression of epinephrine-induced glycogenolysis and activation of glycogen synthase by insulin are sustained in rats fed an HFD similar with those fed a standard chow [31]; 2) mice overexpressing DGAT2 in the liver developed hepatic steatosis, with increased amounts of TG, diacylglycerol, ceramides, and unsaturated long-chain fatty acyl-CoAs in the liver, but had no abnormalities in glucose and insulin tolerance, rates of glucose infusion and hepatic glucose production during hyperinsulinemic-euglycemic clamp studies [32]. These results indicate that hepatic steatosis can occur independently of insulin resistance and raise a ‘beyond triglyceride’ hypothesis. Therefore, searching for toxic lipids that cause hepatic insulin resistance should be required for understanding pathophysiology of fat-induced insulin resistance.

To answer how a toxic lipid causes inflammation and insulin resistance in the liver, we observed the pathophysiology of mice fed an atherogenic diet rich in cholesterol and cholate with or without high fat [33]. One finding was steatohepatitis (Fig. 2A). Cholesterol feeding caused not only steatosis, but also inflammation, ballooning hepatocytes, and fibrosis in the liver. HFD alone caused just mild steatosis without inflammation and fibrosis in the liver, but exacerbated the histological severity of steatohepatitis. Another finding was inflammation. Cholesterol and fat jointly upregulated the genes involved in inflammatory response and p38 mitogen-activated protein kinase signaling pathway, followed by coordinated upregulation of the genes involved in fibrogenesis such as TGF-β-signaling pathway. A third finding was oxidative stress. Oxidative stress markers, protein carbonyls and 4-hydroxy-2-nonenal modified proteins, were accumulated in the liver of mice fed a cholesterol diet, which was further accelerated with HFD (Fig. 2B). Final finding was hepatic insulin resistance. Hepatic expression of insulin receptor substrate (IRS)-2 was downregulated by cholesterol diet (Fig. 2B), probably due to increased SREBP-1c levels [34]. As a result, pyruvate-induced glucose production was exaggerated and a gene for gluconeogenic key enzyme phosphoenolpyruvate carboxykinase (PEPCK) was upregulated.
in these mice [33]. These findings suggest dietary cholesterol as a toxic lipid that causes inflammation and insulin resistance in the liver. High fat feeding itself does not cause steatohepatitis, but accelerates the pathology of steatohepatitis.

3.3. Mitochondrial ROS that mediate fatty acid-induced hepatic insulin resistance

To determine the contribution of FFAs to hepatic insulin resistance, we established an in vitro fatty liver system by incubating H4IIEC3 hepatocytes with a monounsaturated fatty acid (oleate) and a saturated fatty acid (palmitate) (Fig. 3A) and investigated the direct and initial effects of FFAs on hepatocytes [35]. Palmitate, but not oleate, inhibited insulin-stimulated tyrosine phosphorylation of insulin receptor substrate 2 and serine phosphorylation of Akt, through c-Jun NH2-terminal kinase (JNK) activation (Fig. 3B). Among the well-established stimuli for JNK activation, pathways for SREBP-1c and ER stress were unlikely. Instead, mitochondria-derived ROS played a causal role in palmitate-induced JNK activation. In addition, etomoxir, an inhibitor of carnitine palmitoyltransferase-1a (CPT-1a), which is the rate-limiting enzyme in mitochondrial fatty acid β-oxidation, as well as inhibitors of the mitochondrial respiratory chain complex decreased palmitate-induced ROS production. Taken together, our findings in hepatocytes indicate that palmitate accelerates β-oxidation triggered by upregulation of CPT-1 that causes excess electron flux in the mitochondrial respiratory chain, resulting in ROS overproduction. ROS thereby activates JNK that inhibits insulin signal transduction at the level of IRSs (Fig. 3C). Thus, mitochondria-derived ROS induced by palmitate might be major contributors to JNK activation and cellular insulin resistance [35].

4. Mitochondrial oxidativephosphorylation (OXPHOS) and hepatic insulin resistance

4.1. Comprehensive gene expression analyses in the liver of patients with obesity and type 2 diabetes

A comprehensive understanding of gene expression in the liver is a critical step in elucidating the pathology
of diabetes and developing novel therapeutic targets for diabetes. To examine this hypothesis, we undertook a serial analysis of gene expression (SAGE) technique [36] combined with DNA chip analyses in the liver of patients with type 2 diabetes [37] and control subjects with normal glucose tolerance (NGT) [37, 38]. Samples for SAGE were obtained from five patients with type 2 diabetes and five subjects with NGT. We obtained a total of 144,901 tags from the two libraries (NGT, 100,621 tags; type 2 diabetes, 44,280 tags). Many tags were presented repeatedly and a total of 37,054 genes were unique in the two libraries (NGT, 27,622 genes; type 2 diabetes, 15,337 genes). The gene ontologies of cellular components of all identified transcripts were gathered and regrouped into the nine representative classes (extracellular, mitochondria, nucleus, cytoplasm, plasma membrane, ribosome, endoplasmic reticulum, Golgi apparatus, and others) (Fig. 4). The top class for the NGT library was “extracellular,” which includes genes encoding secretory proteins, such as apolipoprotein C-I, apolipoprotein C-III, and albumin. On the other hand, the top class for the type 2 diabetes library was “mitochondria,” which corresponded to a 1.6-fold greater proportion than the NGT library (Fig. 4). The transcripts corresponding to the class “mitochondria” included both
mitochondrial and nuclear transcripts that encode proteins transferred to the mitochondria. For example, the mitochondrial transcripts included 12S rRNA, 16S rRNA, cytochrome c oxidase subunit III, and ATP synthase F0 subunit 6. The nuclear transcripts that encode products transferred to the mitochondria included cytochrome c oxidase subunit VIc, butyryl coenzyme A synthetase 1, and the aldehyde dehydrogenase 2 family.

4.2. Coordinate upregulation of genes involved in OXPHOS pathway in the liver of obese type 2 diabetic patients

Certain features of type 2 diabetes, such as insulin secretory failure and insulin resistance, have been suggested to be caused by mitochondrial dysfunction [39]. It has been reported that skeletal muscle insulin resistance in insulin-resistant children of patients with type 2 diabetes is associated with a dysregulation of intramyocellular fatty acid metabolism. These effects may be due to an inherited defect in mitochondrial oxidative phosphorylation (OXPHOS) [40]. In fact, genes involved in OXPHOS are coordinately downregulated in the skeletal muscle [41], adipose tissue [42], and peripheral blood mononuclear cells [43] of patients with type 2 diabetes. Mitochondrial OXPHOS is not only a major source of ATP, but also a source of reactive oxygen species (ROS) production in most cells [44]. Specifically, electrons passing down the OXPHOS pathway “leak” from the main path and directly reduce oxygen molecules. Thus, as a rule, increased mitochondrial oxygen flux leads to increased formation of ROS [45].

Therefore, we focused on the transcripts involved in OXPHOS [37]. We found 53 nuclear transcripts involved in OXPHOS in the two libraries. Of these transcripts, 36 (68%) were upregulated in the type 2 diabetes library. When comparing the total cumulated tag counts, we found that the overall expression levels of transcripts for OXPHOS were 1.7-fold higher in the type 2 diabetes library (7933 vs. 4748 tags; \( P < 0.00001 \)). SAGE is unique in that it analyzes both nuclear and mitochondrial transcripts. Respiratory complex subunits in the mitochondria are encoded by both nuclear and mitochondrial DNA, and both of these genes involved in OXPHOS were upregulated in the type 2 diabetes library [37]. It has been reported that high ATP levels strongly stimulate mitochondrial rRNA synthesis in isolated human mitochondria [46]. These results suggest that, in the liver of patients with type 2 diabetes, ATP production is activated through the upregulation of OXPHOS. Further, this upregulation stimulates rRNA synthesis and activates energy metabolism. Gluconeogenic enzymes, such as PEPCK, require ATP for their catalytic actions. In addition, the expression levels of representative genes for hepatic gluconeogenesis, such as PGC-1α, TRB-3, gluconeogenic key enzyme mitochondrial PEPCK2, cytosol enzyme PEPCK1, and

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**Fig. 4** The distribution of the cellular components in the type 2 diabetes and the normal glucose tolerance (NGT) libraries

Cellular components of all identified transcripts were gathered by the Gene Ontology consortium (http://www.geneontology.org/). The tag count for the gene was added to the corresponding class. The cumulative tag counts for each class are represented here in tags per million. The top class for the NGT library was “extracellular.” On the other hand, the top class for the type 2 diabetes library was “mitochondria,” corresponding to 1.6 times of that in the NGT library.
bidirectional glucose transporter GLUT2, were significantly upregulated in the type 2 diabetes SAGE library [37]. Upregulation of these genes may provide a molecular explanation for the elevated gluconeogenesis in the livers of patients with type 2 diabetes. To further address the biological pathways leading to the upregulated genes involved in OXPHOS, we explored possible molecular signatures of obesity by analyzing gene expression profiles in the livers of 21 type 2 diabetic patients with (n=10) and without (n=11) obesity using a DNA chip [47]. Metabolic pathways significantly altered by obesity are shown in Table 2 and Fig. 5. Genes involved in glucose metabolism pathways, including the glycolysis, gluconeogenesis, pyruvate metabolism, and tricarboxy-

Table 2  Hepatic expression of representative genes in each metabolic pathway by a DNA chip method in diabetic patients with or without obesity

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Obese/Non-obese</th>
<th>Parametric p-value</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXPHOS</td>
<td>1.33 0.0066</td>
<td>NADH dehydrogenase (ubiquinone) flavoprotein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.25 0.0310</td>
<td>succinate dehydrogenase complex, subunit A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.38 0.0067</td>
<td>ubiquinol-cytochrome c reductase core protein I</td>
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<td></td>
<td>1.33 0.0158</td>
<td>cytochrome c oxidase subunit IV isoform 1</td>
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</tr>
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<td></td>
<td>1.22 0.0301</td>
<td>ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide</td>
<td></td>
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<tr>
<td></td>
<td>1.22 0.0322</td>
<td>ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.63 0.0256</td>
<td>uncoupling protein 2 (mitochondrial, proton carrier)</td>
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</tr>
<tr>
<td></td>
<td>1.22 0.0210</td>
<td>solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 5</td>
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<tr>
<td>Gluconeogenesis</td>
<td>1.50 0.0167</td>
<td>phosphoenolpyruvate carboxykinase 1 (soluble)</td>
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<tr>
<td></td>
<td>1.57 0.0421</td>
<td>glucose-6-phosphatase, catalytic (glycogen storage disease type I, von Gierke disease)</td>
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<td>Fatty acid synthesis</td>
<td>1.50 0.0171</td>
<td>fatty acid synthase</td>
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<td></td>
<td>2.43 0.0161</td>
<td>stearoyl-CoA desaturase (delta-9-desaturase)</td>
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<td>Triglyceride synthesis</td>
<td>1.86 0.0072</td>
<td>diacylglycerol O-acyltransferase homolog 1 (mouse)</td>
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<td></td>
<td>1.63 0.0018</td>
<td>glycerol-3-phosphate dehydrogenase 1</td>
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<td>Beta-oxidation</td>
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<td>carnitine palmitoyltransferase II</td>
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<td>1.50 0.0060</td>
<td>L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain</td>
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<td>1.50 0.0037</td>
<td>peroxisome proliferative activated receptor, alpha</td>
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<td>1.33 0.0114</td>
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Non-obese, non-obese patients with type 2 diabetes; Obese, obese patients with type 2 diabetes.
correlated with insulin resistance indices and the hepatic expression of genes involved in gluconeogenesis, ROS generation (Fig. 5C), and transcriptional factors and nuclear co-activators associated with energy homeostasis, but not with adipocytokines. Collectively, it is hypothesized that an excessive substrate acetyl CoA derived from glucose and fatty acid metabolism flows into the mitochondrial OXPHOS to generate ATP that stimulates gluconeogenic enzymes. In addition, OXPHOS-derived ROS cause hepatic insulin resistance by activating a stress kinase JNK in the liver of patients with type 2 diabetes (Fig. 5D).

However, the causative or compensatory nature of OXPHOS gene expression in the pathogenesis of insu-

4.3. Significance of upregulated OXPHOS pathway in the development of hepatic insulin resistance

The mean expression levels of the OXPHOS pathway calculated as a mean centroid were significantly correlated with insulin resistance indices and the hepatic expression of genes involved in gluconeogenesis, ROS generation (Fig. 5C), and transcriptional factors and nuclear co-activators associated with energy homeostasis, but not with adipocytokines. Collectively, it is hypothesized that an excessive substrate acetyl CoA derived from glucose and fatty acid metabolism flows into the mitochondrial OXPHOS to generate ATP that stimulates gluconeogenic enzymes. In addition, OXPHOS-derived ROS cause hepatic insulin resistance by activating a stress kinase JNK in the liver of patients with type 2 diabetes (Fig. 5D).

However, the causative or compensatory nature of OXPHOS gene expression in the pathogenesis of insu-

chondria where they are used as substrates for OXPHOS to generate ATP. Excessive substrate flux into the mitochondria results in the spin down of electrons and generation of ROS. As estimated, 144 genes encoding proteins for respiratory complexes I, II, III, IV, and V of OXPHOS were coordinately upregulated in the liver in association with obesity (Fig. 5B). Substrate-dependent induction of the enzymes may be involved in upregulation of the genes involved in OXPHOS.

Fig. 5 Obesity upregulates genes involved in oxidative phosphorylation in livers of diabetic patients

Figs. A, B and C are reproduced with permission from Takamura et al. (2008) Obesity (Silver Spring) 16: 2601-2609 [47]
Fatty liver and insulin resistance remains controversial [48]. It appears that in patients with diabetes, OXPHOS gene expression is upregulated in the liver, whereas it is downregulated in skeletal muscle [41, 49]. The difference observed between OXPHOS gene expression in the liver and in skeletal muscle in type 2 diabetic patients may be derived from differences in the glucose uptake systems of the two prevailing cell types, i.e., hepatocytes and myocytes. As hepatocytes have an insulin-independent glucose uptake system, the excessive influx of glucose may easily activate intracellular OXPHOS in the diabetic condition. Indeed, glucose treatment activates OXPHOS in the mitochondria of endothelial cells, which have an insulin-independent glucose uptake system similar to hepatocytes [50]. Thus, in a

![Diagram](image)

**Fig. 5 cont. B** Obesity is associated with the coordinated upregulation of genes involved in the oxidative phosphorylation (OXPHOS) in the livers of patients with type 2 diabetes.

The fold changes presented beside the names of the genes are for the obese versus non-obese patients. The genes significantly ($P < 0.05$) upregulated in obesity are in red; the genes significantly ($P < 0.05$) downregulated are in green. Genes analyzed and not significantly altered in obesity are in gray.

![Diagram](image)

**Fig. 5 cont. C** Hepatic expression of OXPHOS genes clearly correlated with ROS-related genes, suggesting that upregulation of OXPHOS genes is related with ROS generation.
similar manner, OXPHOS gene expression in the liver may be stimulated as a result of the influx of high glucose levels into the hepatocytes. On the other hand, in myocytes with an insulin-dependent glucose uptake system, hyperglycemia may not activate OXPHOS, even in the diabetic condition. Conversely, the upregulation of genes for OXPHOS may cause hyperglycemia since gluconeogenic enzymes, such as PEPCK, require ATP for their catalytic actions. The upregulation of genes for OXPHOS may contribute to hyperglycemia through the increased need for ATP by gluconeogenic enzymes. In addition, the anti-diabetic drug metformin has been reported to halt hepatic gluconeogenesis through the inhibition of the respiratory chain 1 [51, 52]. Our hypothesis appears to be compatible with the pharmacological effects of metformin. Pospisilik et al. [53] demonstrated that liver- or muscle-targeted deletion of AIF decreases mitochondrial OXPHOS and protects mice against obesity and diabetes. Also, in the development of diet-induced obesity in mice, it is reported that mitochondrial dysfunction in the skeletal muscle results from oxidative stress and does not precede the onset of insulin resistance [54]. These findings suggest that coordinate downregulation of genes involved in OXPHOS and mitochondrial dysfunction observed in the skeletal muscles of insulin-resistant humans [41, 49] may be a compensatory response to diabetes rather than a causative factor [48, 53]. Our observation of activated OXPHOS in the liver of patients with obesity was recently confirmed in a study with the tracer experiments for fat and glucose metabolism in human [55]. Subjects with NAFLD have increased adipose lipolysis which contributed to increased lipid delivery to the liver. Hepatic TCA cycle flux is increased, indicating upregulated mitochondrial respiration and increased flux of acetyl-CoA from β-oxidation. Excess acetyl-CoA is selectively partitioned to oxidation in the TCA cycle rather than ketogenesis. Mitochondrial anaplerosis is also increased and provides substrate for the increased rate of gluconeogenesis observed in subjects with fatty liver [55].

Subjects with NAFLD have elevated adipose lipolysis which contributed to increased lipid delivery to the liver. Hepatic TCA cycle flux is increased, indicating upregulated mitochondrial respiration and increased flux of acetyl-CoA from β-oxidation. Excess acetyl-CoA is selectively partitioned to oxidation in the TCA cycle rather than ketogenesis. Mitochondrial anaplerosis is also increased and provides substrate for the increased rate of gluconeogenesis observed in subjects with fatty liver [55]. Such selective flow of the substrate depending on increased energy requirements of the fatty liver may increase hepatic gluconeogenesis in patients with obesity. These findings suggest that the upregulation of genes for OXPHOS in the liver may contribute to selective hepatic insulin resistance in type 2 diabetes: insulin fails to suppress gluconeogenesis but continues to activate lipogenesis [56].
5. Searching for a novel hepatokine that leads to the pathophysiology of type 2 diabetes

5.1. A concept of hepatokines

One of the links among key organs to maintain energy homeostasis may be a hormone that is defined as a secretory protein acting as a signaling molecule in other cells, and altering their growth, function or metabolism. In addition to the classical endocrine organs such as pituitary gland, thyroid gland, adrenal gland, ovary, and testes, it has been recognized that most cells are capable of producing various molecules serving as a hormone. Indeed, adipose tissue functions as a key endocrine organ by releasing adipokines that have pro-inflammatory or anti-inflammatory activities. Accumulating evidence has led to the establishment of a concept that dysregulated production or secretion of these adipokines owing to adipose tissue dysfunction can contribute to the pathogenesis of obesity-linked complications. Such so-called adipocytes are derived from fibroblasts (Fig. 6). If an adipocyte is defined as a cell in which lipid droplets accumulate in the face of an overnutrition state, and is not limited to fibroblasts, but also includes hepatocytes and myocytes, the latter can function as an adipocyte and may also contribute to the pathophysiology of obesity and its associated diseases (Fig. 6). Specifically, our comprehensive gene expression analyses revealed that genes coding extracellular proteins are abundantly expressed in human liver tissue (Fig. 4) [36, 37].

Because C-reactive protein is mainly produced in the liver in response to acute myocardial infarction, we hypothesized that the liver plays a pivotal role in the pathophysiology of acute coronary syndrome. Genes related to tissue remodeling, adhesion molecules, and morphogenesis were significantly upregulated in the livers of mice with myocardial ischemia/reperfusion or infarction but not in those with liver ischemia/reperfusion [57]. Detailed analysis of the signaling pathways suggested that osteopontin released from the heart altered the signaling pathways of the livers of mice under myocardial ischemia. Moreover, osteopontin stimulated primary hepatocytes to secrete vascular endothelial growth factor-A, which is important for tissue remodeling [57]. Therefore, hepatic gene expression is potentially regulated by cardiac humoral factors under myocardial ischemia and may thereby rescue the ischemic heart, suggesting the existence of the liver-organs networks to maintain body homeostasis.

By using an in-house cDNA microarray, we found that hepatic expression of genes encoding angiogenic factors, fibrogenic factors, and redox-associated factors are altered in people with type 2 diabetes compared with those without diabetes [25, 58, 59]. This differential expression may contribute to the pathophysiology of type 2 diabetes and its clinical manifestations.

![Fig. 6](image)

Fig. 6 Steatosis of cell may contribute to pathophysiology of obesity and type 2 diabetes

So-called adipocytes are derived from fibroblasts. If an adipocyte is defined as a cell in which lipid droplets accumulate, and is not limited to fibroblasts, but can also include hepatocytes, the latter can become an adipocyte, and may also contribute to the pathophysiology of obesity and type 2 diabetes.
Based on these findings, we hypothesized that, in a manner analogous to adipose tissues [60], the liver may also contribute to energy homeostasis by way of the production of secreted proteins, termed hepatokines, and that the dysregulation of hepatokines contributes to the pathophysiology of diabetes and subsequent complications.

5.2. Strategies for identifying a hepatokine

To identify a novel hepatokine, we first applied a SAGE technique [36] that makes it possible to compare tag levels among independent libraries and to identify previously unrecognized functional hepatic genes that may regulate the pathophysiology of diabetes [37] as described above. Most abundant mRNAs in the liver were genes for secretory proteins, suggesting that the liver is a major source of secretory proteins [36, 37]. We identified 117 genes encoding putative secretory proteins with expression levels at least 1.5-fold higher in diabetic patients than compared with normal subjects. Second, of these candidate genes, DNA chip methods were used to identify genes whose hepatic expression levels were significantly correlated with glycemic control (HbA1c), obesity (BMI), or insulin resistance (HOMA-R and metabolic clearance rate). Third, we referred the expression of the genes to the various animal models of diabetes, obesity, and fatty liver [15, 27, 33, 61]. Based on these approaches, we isolated 62 candidate genes for hepatokines associated with insulin resistance, hyperglycemia, and obesity [36]. Of these, we identified a gene encoding selenoprotein P (SeP), the expression levels of which were positively correlated with insulin resistance and hyperglycemia [62]. Indeed, the serum levels of SeP were elevated in people with type 2 diabetes, and significantly correlated with fasting plasma glucose and HbA1c levels [62]. Furthermore, serum SeP levels are reported to be associated with cardiometabolic factors including BMI, waist circumference, systolic blood pressure, triglycerides, and aspartate aminotransferase, carotid intima-media thickness and high-sensitivity C-reactive protein [63].

5.3. Selenoprotein P as a diabetes-associated hepatokine causes systemic insulin resistance

SeP is a 50 kDa secretory protein mainly produced in the liver, and has been known to be a selenium-carrier protein that distributes selenium to peripheral tissues such as brain and testis [64]. However, its metabolic actions had remained unknown.

In a hyperinsulinemic euglycemic clamp study, administration of purified SeP enhanced hepatic glucose production and suppressed glucose uptake into the skeletal muscle in C57BL mice. Insulin-stimulated Akt phosphorylation was impaired in liver and skeletal muscle of mice pretreated with SeP. Hepatic expression of Sepp 1 and serum levels of SeP were increased by approximately 1.5 fold in type 2 diabetic model OLETF rats and KKAY mice compared with the controls. A liver- and blood-specific 30% reduction of SeP protein levels by delivery of Sepp1-specific siRNAs into KKAY mice using a hydrodynamic transfection method improved both glucose intolerance and insulin resistance in KKAY mice, suggesting that SeP can be a therapeutic target for insulin resistance. Indeed, mice deleted with selenoprotein P was insulin sensitive. Insulin-induced phosphorylation of Akt was enhanced in the liver and skeletal muscle of SeP knockout mice [62]. These findings indicate that a liver-derived SeP causes insulin resistance in the liver and skeletal muscle.

5.4. Regulation of selenoprotein P expression by nutrients for energy homeostasis

Concerning regulation of SeP by nutrients, glucose and palmitate upregulates and insulin downregulates Sepp1 expression in H4IIEC hepatocytes. Hepatic Sepp1 mRNA was elevated in fasting condition in mice possibly due to a decreased level of insulin and an elevated level of fatty acids [62]. Therefore, the regulation of SeP by nutrients seems consistent with the glucose homeostasis in the body; i.e., in the fasting state, lower level of insulin and elevated level of fatty acids induce SEPP1 expression (Fig. 7). SeP reduces insulin sensitivity leading to enhanced hepatic glucose output and diminished glucose uptake into the skeletal muscle, and thereby maintains circulating glucose levels. The nutrient-sensitive promoter regions of SEPP are under investigation.

5.5. AMP-activated protein kinase (AMPK) as a target of selenoprotein P to cause insulin resistance

Concerning the molecular mechanisms underlying SeP-induced insulin resistance, we found that treatment with SeP reduces phosphorylation of AMPK and expression of fatty acid beta oxidation-related genes in H4IIEC hepatocytes. AMPK is a key player in anti-aging by activating PGC-1α and FoxO to enhance energy
expendsiture and longevity. Constitutively-active AMPK canceled SeP-induced insulin resistance in H4IIEC hepatocytes, suggesting that SeP causes insulin resistance, at least partly, by inactivating AMPK [62] (Fig. 7).

An inhibitory effect of SeP on AMPK seems opposite to the effects of adiponectin and metformin. In concert with these assumptions, circulating SeP levels were negatively associated with serum levels of both total and high-molecular adiponectin in patients with type 2 diabetes [65]. SeP was a predictor of both total and high-molecular adiponectin levels, independently of age, body weight, and quantitative insulin sensitivity index. In addition, SEPP1- knockout mice exhibited an increase in serum adiponectin levels when fed regular chow or a high sucrose, HFD. These results suggest that overproduction of SeP is connected with hypoadiponectinemia in patients with type 2 diabetes [65]. Also, we have found that antidiabetic metformin directly acts on the promoter of SEPP1 and downregulates SEPP1 gene expression (unpublished data).

5.6. Possible reductive stress underlying selenoprotein P-induced insulin resistance

Specifically, SeP acts as a redox protein by activating glutathione peroxidase. How does anti-oxidant SeP induce insulin resistance? Indeed, selenium supplementation was paradoxically associated with an increased risk for diabetes in humans [66]. Also, in our previous study in a cultured hepatocyte cell line, anti-oxidant reagents, N-acetyl-L-cysteine, rescued palmitate-induced insulin resistance only partly, whereas it effectively suppressed palmitate-induced activation of JNK [35]. To solve this paradox, we addressed the concentration-dependent effects of ROS on insulin signaling in hepatocytes. Treatment with high concentrations of H$_2$O$_2$ reduced insulin-stimulated Akt phosphorylation by activating JNK, whereas lower concentrations of H$_2$O$_2$ enhanced insulin-stimulated phosphorylation of Akt by suppressing PTP1B activ-

![Image: Diagram showing the relationship between SeP and insulin resistance](Fig. 7) SeP as a diabetes-associated hepatokine that causes systemic insulin resistance

Hepatic overproduction of SeP causes insulin resistance in patients with obesity and/or type 2 diabetes. Decreased insulin action and excessive glucose or fatty acid uptake upregulate SEPP1 mRNA in the liver, leading to the elevation of circulating SeP levels. SeP induces insulin resistance, at least partly, by inactivating in an autocrine/paracrine manner in the liver.
Collectively with these findings and our SeP story [62], there may be a pitfall in anti-oxidant supplements for the treatment of diabetes/obesity-associated diseases. Future diabetes research may involve possible reductive stress, together with oxidative stress, to cause insulin resistance.

Indeed, similar to the Sepp1-knockout mice, mice lacking one of the selenoproteins involved in the elimination of physiological ROS, glutathione peroxidase 1, are reported to be protected from HFD-induced insulin resistance [68]. Furthermore, supplementation with antioxidants may preclude health-promoting effects of physical exercise in humans [69]. Consistent with the concept of mitohormesis, exercise-induced oxidative stress ameliorates insulin resistance and causes an adaptive response promoting endogenous antioxidant defense capacity.

Collectively with these findings and our SeP story [62], there may be a pitfall in anti-oxidant supplements for the treatment of diabetes/obesity-associated diseases. Future diabetes research may involve possible reductive stress, together with oxidative stress, to cause insulin resistance.

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