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Molecular Mechanisms and Therapeutic Targets in Steatosis and Steatohepatitis

NORA ANDERSON AND JÜRGEN BORLAK

Medical School of Hannover, Center Pharmacology and Toxicology, Hannover, Germany; and Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany

Address correspondence to: Prof. Dr. Jürgen Borlak, Fraunhofer Institute of Toxicology and Experimental Medicine, Nikolai-Fuchs-Str. 1, 30625 Hannover, Germany. E-mail: borlak@item.fraunhofer.de

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Abstract——**Steatosis of the liver may arise from a variety of conditions, but the molecular basis for lipid droplet formation is poorly understood. Although a certain amount of lipid storage may even be hepatoprotective, prolonged lipid storage can result in an activation of inflammatory reactions and loss of metabolic competency. Apart from drug-induced steatosis, certain metabolic disorders associated with obesity, insulin resistance, and hyperlipidemia give also rise to nonalcoholic fatty liver diseases (NAFLD). It is noteworthy that advanced stages of nonalcoholic hepatic steatosis and steatohepatitis (NASH) result ultimately in fibrosis and cirrhosis. In this regard, the lipid droplets (LDs) have been discovered to be metabolically highly active structures that play major roles in lipid transport, sorting, and signaling cascades. In particular, LDs maintain a dynamic communication with the endoplasmic reticulum (ER) and the plasma membrane via sphingolipid-enriched domains of the plasma membrane—the lipid rafts. These microdomains frequently harbor receptor tyrosine kinases and other signaling molecules and connect extracellular events with intracellular signaling cascades. Here, we review recent knowledge on the molecular mechanisms of drug and metabolically induced hepatic steatosis and its progression to steatohepatitis (NASH). The contribution of cytokines and other signaling molecules, as well as activity of nuclear receptors, lipids, transcription factors, and endocrine mediators toward cellular dysfunction and progression of steatotic liver disease to NASH is specifically addressed, as is the cross-talk of different cell types in the pathogenesis of NAFLD. Furthermore, we provide an overview of recent therapeutic approaches in NASH therapy and discuss new as well as putative targets for pharmacological interventions.**

I. Introduction: Hepatic Steatosis—A Common Road with Different Entrances

The term hepatic steatosis (fatty liver) refers to an intracellular accumulation of lipids and subsequent formation of lipid droplets (LD[1]) in the cytoplasm of hepatocytes that is associated with an enlargement of the liver (hepatomegaly). When steatosis of the liver is further accompanied by inflammation, the condition is termed steatohepatitis. Both pathological conditions are subsumed under the term of nonalcoholic fatty liver disease (NAFLD) if alcohol can be excluded as a primary cause. Thus, NAFLD refers to steatosis as well to its progressive stages [i.e., steatohepatitis (NASH) and fatty liver-associated cirrhosis].

Here, we wish to review mechanisms resulting in NAFLD and NASH and focus particularly on the molecular and cellular basis of lipid droplet formation in steatosis; the role of individual organelles, such as the ER, peroxisomes, lysosomes, mitochondria; and biochemical reactions associated with steatosis of the liver. Furthermore, we highlight the molecular events in the switch from steatosis to steatohepatitis and the associated cross-talk among stellate cells, macrophages, endothelium of the sinusoid, and fibroblasts, among others. We also address the role of fatty acids (FA) and phospholipids as lipotoxic agents in NASH and discuss recent insight into the pathophysiology of drug-induced steatosis and steatohepatitis. Finally, this review summarizes findings from therapeutic interventions in the treatment of NAFLD.

It is noteworthy that in the Western population, overnutrition is the most common cause of NAFLD, with an estimated incidence of 15 to 20%, and an increasing number of patients presenting risk factors for its development (Bedogni et al., 2005; Amarapurkar et al., 2007; Zhou et al., 2007a,b). Overnutrition- and obesity-related NAFLD is a multifactorial disorder and linked to hypertriglyceridemia, obesity, and insulin resistance, as ob-

[1] Abbreviations: LD, lipid droplet; 5-LO, 5-lipoxygenase; ADRP, adipose differentiation-related protein; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; ApoB, apolipoprotein B; ATII, angiotensin II; BMI, Body mass index; CAR, constitutive active/androstane receptor; ChREBP, carbohydrate response element-binding protein; COX, cyclooxygenase; CPT, carnitine palmitoyltransferase; DGAT, acyl-CoA:diacylglycerol acyltransferase 2; DHA, docosahexaenoic acid; EAR, v-*erbA*-related protein; ERK1/2, extracellular signal-regulated kinase 1/2; FA, fatty acid; FAS, fatty acid synthase; FFA, free fatty acids; FXR, farnesoid X receptor; FoxO1, Forkhead box O1; GGT, γ-glutamyl transferase; GLUT4, glucose transporter 4; HNF, hepatic nuclear factor; HSL, hormone-sensitive lipase; ICAM, intercellular adhesion molecule; IKK-β, IκB-β kinase; IL, interleukin; iNOS, inducible nitric-oxide synthase; IR, insulin resistance; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; LCACoA, long-chain acyl-CoA; LCFA, long-chain fatty acid; LDAP, lipid droplet-associated protein; LPC, lysophosphatidylcholine; LPS, lipopolysaccharide; LXR, liver X receptor; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MCD, methionine- and choline-deficient; MCP-1, monocyte-chemoattractant protein 1; MODY1, maturity-onset diabetes of the young type 1; MRC, mitochondrial respiratory chain; MTP, mitochondrial triglyceride transfer protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NEFA, nonesterified fatty acid; NF-κB, nuclear factor-κB; NO, nitric oxide; OXPHOS, oxidative phosphorylation; PAT, perilipin, adipophilin, and TIP47; PC, phosphocholine; PE, phosphoethanolamine; PGE, prostaglandin E; PI3K, phosphatidyl inositol-3 kinase; PK, protein kinase; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; RXR, retinoid X receptor; SPMase, sphingomyelinase; SPT, serine palmitoyltransferase; SRE, sterol regulatory element; SREBP-1, sterol regulatory element binding protein; T2DM, type 32 diabetes mellitus; TAG, triacylglycerol; TF, transcription factor; TLR, TOLL-like receptor; TNF-α, tumor necrosis factor α; UCP, uncoupling protein; VLDL, very-low-density lipoprotein; WY-14643, pirinixic acid.

served in patients with metabolic syndrome (Higuchi and Gores, 2003). It has been suggested that hepatic steatosis should be considered part of the metabolic syndrome and that insulin resistance is a prerequisite for its development (Marchesini et al., 2001a). The prevalence of steatosis in patients with obesity is about 75% (Browning et al., 2004; Adams et al., 2005). It is remarkable that nearly 35% of these develop NASH (Ong et al., 2005; Xanthakos et al., 2006). Insulin resistance as observed in overnutrition and obesity is thought to be inevitably linked to the pathogenesis of NAFLD, which throughout the text will be referred to as "primary" NAFLD. In contrast, development of "secondary" fatty liver diseases includes other factors, such as exposure to drugs and xenobiotics, but also parenteral nutrition and surgical interventions [e.g., liver transplantation and jejunoileal bypass surgery (Pessayre et al., 2001)]. When alcohol is involved, the condition is referred to as alcohol-related fatty liver disease and needs to be distinguished from NAFLD (Ludwig et al., 1980; Pessayre et al., 2002). Other secondary causes, such as virus-induced NAFLD (e.g., hepatitis C) have been subject of other reviews (Negro, 2006; Bongiovanni and Tordato, 2007). It is of great importance that a considerable fraction of patients diagnosed with steatohepatitis progresses to advanced stages of disease (i.e., fibrosis and cirrhosis, the latter of which has been associated with the development of hepatocellular carcinoma) (Bugianesi et al., 2002; Fassio et al., 2004). Furthermore, an increased incidence of NAFLD has been reported for patients diagnosed with type 2 diabetes, further documenting the need for an improved understanding of the pathogenesis of NAFLD (McGarry, 2002; Targher et al., 2007).

Indeed, the molecular events resulting in intrahepatic lipid accumulation and growth of lipid droplets are poorly understood, but may arise from 1) increased uptake of lipids, 2) elevated de novo synthesis of fatty acids, 3) impaired lipoprotein synthesis or secretion, and/or 4) reduced fatty acid oxidation (Chitturi and Farrell, 2001; Charlton et al., 2002; Bradbury and Berk, 2004; Grieco et al., 2005; Farrell and Larter, 2006). In this regard, the liver plays a central role in the energy homeostasis by storing glucose as glycogen and distributing fuels in the form of glucose and lipids to peripheral organs. Hepatic glucose and free fatty acid uptake occurs in an insulin-independent fashion and is basically thought to increase linearly with the postprandial rise in plasma concentrations (Bradbury and Berk, 2004). Dietary lipids in the form of chylomicrons are transported from the gut via the lymphatic system to the liver, where they are incorporated after release from lipoproteins by hepatic lipoprotein lipase (Bradbury and Berk, 2004). Physiologically and during the postprandial phase, dietary lipids are stored in the liver, where they are processed and assembled with apolipoprotein B 100 (ApoB) to form very-low-density lipoprotein (VLDL). These particles are secreted and distribute lipids to lipid-storing adipose tissue (Bradbury and Berk, 2004). Consequently, dietary overload with both lipids and glucose may result in hepatic lipid accumulation (Bray et al., 2004). Although an excess supply of carbohydrates results in insulin-dependent de novo fatty acid synthesis from acetyl-CoA, high-fat diets result in increased hepatic lipid storage.

In patients with NAFLD, the intrahepatic triacylglycerol content seems to depend mainly on the systemic availability of free fatty acids. This was suggested by isotope labeling studies in which serum free fatty acids were shown to account for 59% of hepatic TAG (Donnelly et al., 2005). Elevated levels of circulating free fatty acids observed in patients with NASH and in insulin-resistant patients are explained by a loss of sensitivity to insulin in adipose tissue that is associated with a failure to suppress lipolysis (Sanyal et al., 2001; de Almeida et al., 2002). Although the liver is the major organ for lipid distribution, the hepatic capacity to store lipids is limited, and only relatively small amounts of intrahepatic lipids are thought to critically influence the metabolic competence of the liver (Szczepaniak et al., 1999; Petersen et al., 2005). Thus, an insufficiency in peripheral lipid storage possibly arising from different pathological conditions (i.e., in peripheral insulin resistance, chronic inflammation, or lipodystrophy) may result in a hepatic overflow of lipids and subsequently hepatic steatosis (Lewis et al., 2002; Roden, 2006).

Furthermore, there is evidence that rate of de novo lipid synthesis is elevated in livers of (insulin-resistant) patients with NAFLD, compared with healthy subjects (Schwarz et al., 2003; Donnelly et al., 2005). A shift from fatty acid oxidation to de novo lipid synthesis is mediated by an increased activity of the transcription factors peroxisome proliferator-activated receptor (PPAR) γ (Schadinger et al., 2005), carbohydrate response element-binding protein (ChREBP), and sterol regulatory element-binding protein-1c (SREBP-1c) (Shimomura et al., 1999; Yahagi et al., 1999), all of which are positive modulators of hepatic triglyceride contents by targeting genes coding for key reactions in lipid synthesis (Dentin et al., 2006). Undue activation of lipogenic transcription factors during excess supply of dietary lipids was observed in insulin resistant states (for details, see section II.A).

In addition, steatosis and its progression to steatohepatitis may result from improper fatty acid oxidation, as observed in patients carrying variant alleles of mitochondrial acyl-CoA dehydrogenases and on the basis of studies with transgenic mice with deficiencies in mitochondrial fatty acid oxidation (Tolwani et al., 2005; Grosse et al., 2006; Zhang et al., 2007). In primary NAFLD, the intracellular accumulation of intermediary products of fatty acid synthesis, such as malonyl-CoA, may negatively affect fatty acid transport into mitochondria and its oxidation by inhibiting carnitine palmitoyl-

transferase (CPT-1), the rate-limiting enzyme in mitochondrial fatty acid uptake (Bandyopadhyay et al., 2006; Lavoie and Gauthier, 2006). Whereas in secondary NAFLD, such as drug-induced hepatic steatosis, inhibition of mitochondrial fatty acid oxidation is thought to be a major cause for intrahepatic lipid accumulation (Reddy and Rao, 2006). It has been further suggested that impaired hepatic lipid clearance via VLDL may be a possible cause of hepatic lipid accumulation in NAFLD (Sparks et al., 1997; Zhang et al., 2004).

Taken collectively, there is no single causal explanation for the development of primary hepatic steatosis; the evidence so far indicates that an interplay of different factors may be responsible for the onset and progression to NASH. This raises the question of why certain individuals are at risk of developing hepatic steatosis and subsequently NASH. It is noteworthy that studies in different ethnic populations have demonstrated that prevalence of steatosis in African-American patients is much lower compared with Hispanic or non-Hispanic white patients diagnosed with metabolic syndrome, suggesting an involvement of additional factors, besides those associated with the metabolic syndrome, in determining the development of NAFLD (Caldwell et al., 2007). In this regard, carriers of variant alleles of the hemochromatosis (HFE) gene (Valenti et al., 2003; Walsh et al., 2006), the PPARγ coactivator-1, sterol regulatory element–binding protein gene-1 (Eberlé et al., 2004), hepatic lipase (Stefan et al., 2005), and mitochondrial triglyceride transfer protein (MTP) (Gambino et al., 2007) were proposed to be at added risk for NAFLD (Pessayre, 2007).

Besides obscurities regarding genetic risk factors contributing to hepatic lipid accumulation and the development of hepatic steatosis, one of the most exciting questions in NAFLD is why and how intrahepatic lipid accumulation leads to the development of inflammation. Factors responsible for the switch from steatosis to steatohepatitis have been the subject of extensive research and speculation.

The initially proposed "two-hit" model by Day and James (1998) provides a pathophysiologic rationale for the progression to steatohepatitis, claiming that the reversible intracellular deposition of triacylglycerols (TAG) ("first hit") leads to metabolic and molecular alterations that sensitize the liver to the second "hit," usually referred to as oxidative stress and cytokine-induced liver injury. It is noteworthy that, based on longitudinal studies, one third of patients with hepatic steatosis are estimated to progress to steatohepatitis (Harrison et al., 2003b). This stage of disease is characterized by cumulating defects in cellular organelles (e.g., mitochondria), elevated systemic and local levels of cytokines, recruitment of macrophages, as well as subsequent changes leading to the remodelling process of the intracellular matrix, which paves the way to fibrosis and possibly cirrhosis (Farrell and Larter, 2006; Pessayre, 2007). In fact, lipid accumulation and short- and long-term exposures to NEFAs have been demonstrated to result in detrimental effects in vitro and in vivo, including increased generation of oxidative stress, induction of cellular stress responses [e.g., activation of protein kinase C (PKC), mitogen-activated protein kinase (MAPK), jun N-terminal kinase (JNK), nuclear factor-κB (NF-κB)], and subsequent expression of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) (Reddy and Rao, 2006). In particular, fatty acids are believed to agitate the progress of steatohepatitis by inducing cellular stress and organelle toxicity of fatty acids (Diehl, 2005).

Activation of stress-related signal cascades subsequently induces down-regulation of the cellular energy metabolism and alleviation of insulin sensitivity, thereby enhancing further intracellular accumulation of lipids, finally turning to an irreversible condition. Despite the collective knowledge that has been gathered over the last few decades, it remains unclear which factors trigger the disequilibria between pro- and anti-inflammatory pathways, ultimately turning a reversible accumulation of lipid droplets into an irreversible and progressive condition.

Taken collectively, steatosis of the liver may arise from an excess supply of fatty acids and/or glucose, lipotoxicity, and insulin resistance; its progression to NASH is inevitably linked to the paracrine effect of pro-inflammatory cytokines and imbalanced adipokines. Figure 1 depicts some basic events involved in pathogenesis of NASH: an increased pool of free fatty acids induces de novo lipid synthesis by activation of nuclear receptors SREBP-1, ChREBP-1, and PPARγ. Elevated production of reactive oxygen species (ROS) contributes to organelle toxicity, suppression of fatty acid oxidation, and an increase in lipid peroxidation. Furthermore, inhibition of lipoprotein assembly and secretion may contribute to intracellular accumulation of triacylglycerols (Fig. 1).

Cytokines are involved in the recruitment and activation of Kupffer cells and the transformation of stellate cells to the fibromyoblastic cell types, both of which have been found to contribute to the progression from steatosis to steatohepatitis (Bilzer et al., 2006). Furthermore, pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, have been shown to affect insulin signaling and therefore might play a role in the development of insulin resistance (Bugianesi et al., 2002). In addition, adipokines such as adiponectin, leptin, and resistin have been implicated in the pathogenesis of NAFLD by modulating insulin resistance and lipid oxidation rates. There is evidence for adiponectin and resistin levels to be negatively correlated with hepatic lipid accumulation and grade of inflammation in NASH, but the role of leptin in early states of the disease remains inconclusive (Tsochatzis et al., 2006; Ikejima et al., 2007). It is noteworthy that leptin was reported to play a role in the progression to fibrosis. Below, we review recent knowledge on he-

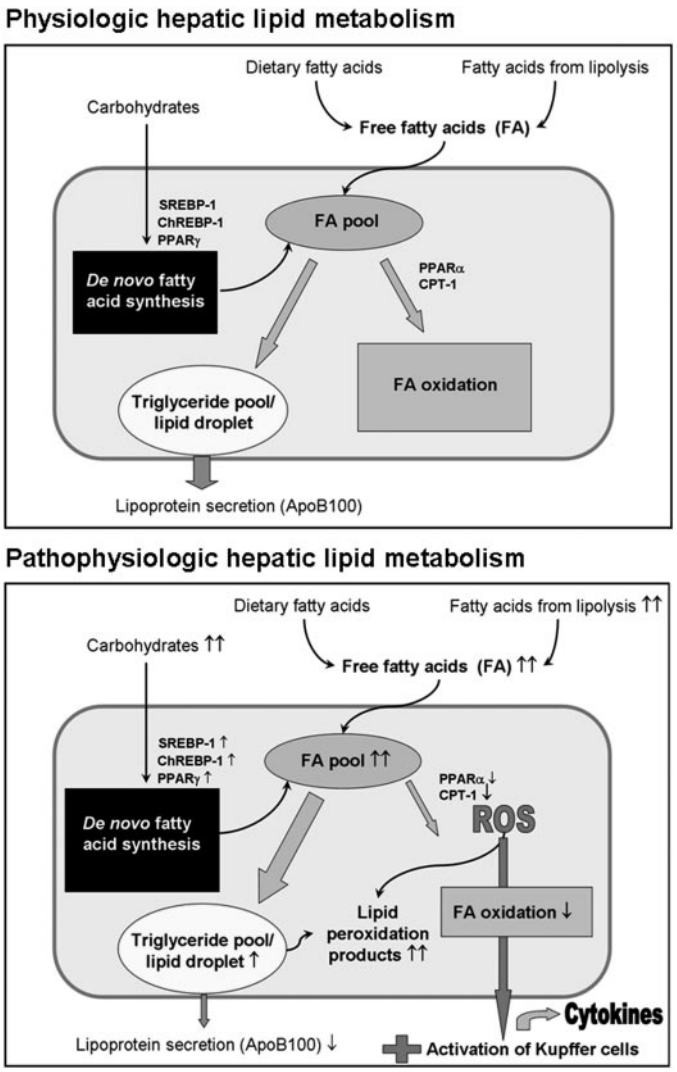

FIG. 1. Pathogenic mechanisms in metabolically induced NASH. Under physiological conditions, uptake, storage, and excretion of lipids is balanced (top); increased fatty acid supply and reduced lipid clearance trigger fatty acid esterification and their storage in the form of lipid droplets in hepatocytes (bottom).

patic steatosis and steatohepatitis, the concept of lipotoxicity, and a selection of targets presumably involved in organelle dysfunction in fatty liver disease. Furthermore, we will highlight the genesis of lipid—lipid droplet formation and its activity in cell signaling, intracellular lipid trafficking, and channelling toward NAFLD.

II. Molecular Mechanisms in the Pathogenesis of Nonalcoholic Hepatic Steatosis and Steatohepatitis

A. Impaired Insulin Signaling and Reduced Insulin Sensitivity as a Molecular Cause for Steatosis?

Insulin resistance is associated with a defect in insulin signaling and results in metabolic defects of both glucose and lipid metabolism. Insulin resistance precedes type 2 diabetes and the connection between hepatic steatosis and insulin resistance has been established in many individual experimental settings. Nonetheless, a *causal relationship* between hepatic lipid accumulation and insulin resistance (IR) remains to be established (Pan et al., 1997; Gavrilova et al., 2000; Lewis et al., 2002; Luzi et al., 2003).

To better understand the connection between insulin resistance and hepatic steatosis, the physiologic role of insulin will be briefly described. In peripheral tissues, including adipose tissue and skeletal muscle, postprandial secreted insulin initiates the translocation of glucose transporter 4 (GLUT4) transporters from intracellular vesicles to the plasma membrane, thereby enabling glucose uptake and utilization. Another important peripheral effect of insulin is its lipogenic effect and suppression of peripheral lipolysis (hormone-sensitive lipase). Upon binding to the dimeric insulin receptor, autophosphorylation of the receptor takes place with subsequent tyrosine phosphorylation of the adaptor protein insulin receptor substrate (IRS) 1 and activation of phosphatidyl inositol-3 kinase (PI3K) (Chang et al., 2004). Elevated postprandial insulin levels control blood sugar concentrations through inhibition of gluconeogenesis and stimulation of glycogen synthesis. Inhibition of gluconeogenesis is mainly achieved by suppression of the gluconeogenic key enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (Saltiel and Kahn, 2001). Furthermore, insulin enhances hepatic de novo lipid synthesis by activation of lipogenic transcription factors (Horton et al., 2003a; Denechaud et al., 2008). At the same time, induction of the lipogenic enzyme acetyl-CoA carboxylase leads to conversion of acetyl-CoA to malonyl-CoA, which inhibits mitochondrial fatty acid oxidation, thereby lowering combustion of fatty acids. Thus, glucose is redirected to glycogen pools, and excess glucose is used in de novo lipogenesis. It is noteworthy that the insulin signaling pathway has been the subject of general reviews (Saltiel and Kahn, 2001; Chang et al., 2004).

In brief, insulin binding to the hepatic insulin receptor initiates phosphorylation of IRS-1 and IRS-2. The latter proteins are recognized by Src homology 2 domain of the p85 regulatory subunit of PI3K, which ultimately leads to activation of AKT signaling cascades via release of phosphatidylinositol 3,4,5-trisphosphate. Downstream of IRS-1 and IRS-2, glycogen synthesis is activated by AKT-dependent phosphorylation of glycogen synthase kinase 3, whereas further effects on glucose, protein, and lipid metabolism are mediated via various signaling molecules [i.e., mammalian target of rapamycin, MAP/extracellular signal-regulated kinase kinases, cAMP-specific phosphodiesterases, and regulation of gene expression through the transcription factors SREBP-1, FOXO1, and FOXA2 among others (Saltiel and Kahn, 2001)].

Insulin resistance (an impaired response of tissues upon insulin stimulation) is observed in obesity, in the metabolic syndrome, and in patients with NAFLD and

has been observed in peripheral tissues as well as the liver (McGarry, 2002). Peripheral insulin resistance results in enhanced lipolysis and impaired lipid storage as a result of reduced inhibition of hormone-sensitive lipase (HSL) and reduced activity of transcription factors involved in lipid droplet formation, such as PPARγ (Bradbury and Berk, 2004; Guilherme et al., 2008), whereas hepatic insulin resistance may result in insufficiently suppressed gluconeogenesis (Blaak et al., 2000).

Aberrations of insulin signaling cascades have been linked to atypical phosphorylation of serine residues at the level of IRS-1 and IRS-2, which prevents proper tyrosine phosphorylation and, consequently, activation of downstream signaling molecules (Mlinar et al., 2007). Novel PKCs have been suggested to be involved in atypical phosphorylation of IRS-1 and IRS-2 and subsequent failure of insulin signaling. For example, PKC-θ (Griffin et al., 1999; Yu et al., 2002) and PKC-ε in rodents (Samuel et al., 2004, 2007) and PKC-δ and -βII in humans (Itani et al., 2002) were implicated in the pathogenesis of insulin resistance of skeletal muscle, whereas Lam et al. (2002) and Samuel et al. (2004) particularly suggested the isoforms PKC-ε and PKC-δ to be possible mediators of hepatic insulin resistance (.

Defects in insulin signaling and peripheral insulin resistance have been linked to intramyocellular and intrahepatocellular lipid accumulation (McGarry, 2002). Such imaging studies had evidenced a particularly tight inverse correlation between intrahepatic triglyceride contents and insulin sensitivity measured by whole-body glucose disposal during euglycemic-hyperinsulinemic clamp studies (Hwang et al., 2007; Korenblat et al., 2008). Indeed, intrahepatic triglyceride contents predicted insulin sensitivity in liver, skeletal muscle, and adipose tissue better than body mass index (BMI) or body fat (Korenblat et al., 2008). Consequently, reduction of intrahepatic lipid contents ($\sim$80%) by a moderately hypocaloric very-low-fat diet (3%) in patients with obesity and T2DM enhanced insulin sensitivity by normalizing insulin suppression of hepatic glucose production but had no effects on peripheral insulin sensitivity (Petersen et al., 2005).

There is evidence for lipid-induced suppression of insulin signaling via IRS-1 and IRS-2 to be responsible for peripheral insulin resistance (Savage et al., 2005). The pathogenic connection between free fatty acids, hepatic steatosis, and insulin resistance was established in diverse animal models in which high-fat diets were found to induce not only steatosis and steatohepatitis but also whole-body and hepatic insulin resistance (McGarry, 2002). Roden et al. (1996) were the first to demonstrate that elevated free fatty acid levels in humans were able to decrease insulin sensitivity. These experiments demonstrated that infusion of free fatty acids resulted in inhibition of glucose transport and phosphorylation, which was followed by a reduction of approximately 50% in the rate of muscle glycogen synthesis and glucose oxidation.

Short-term feeding of high-fat diets resulted in hepatic triacylglycerol accumulation and insulin resistance in the treated animals, as determined by a dramatically diminished suppression of gluconeogenesis of 8 versus 74% in treatment groups and control groups, respectively. The specific relationship between hepatic fat accumulation and hepatic insulin resistance in this study was evident in an impairment of insulin-stimulated IRS-1 and IRS-2 tyrosine phosphorylation in the group of fat-fed animals. This subsequently resulted in an impaired activation of AKT2 and inactivation of glycogen synthase kinase 3. Stimulation of mitochondrial fatty acid oxidation by the mitochondrial uncoupler 2,4-dinitrophenol reduced hepatic accumulation and abrogated hepatic insulin resistance (Samuel et al., 2004).

Such mitochondrial uncoupling results in an elevation of long-chain acyl-CoA (LCACoA) and the formation of diacylglycerol, which in turn activates serine kinases, such as PKC-θ. This hypothesis was supported by the finding that restoration of insulin sensitivity in skeletal muscle of rats fed a high-fat diet was associated with simultaneous reduction in muscle LCACoA levels and translocation of PKC-θ from the plasma membrane to the cytoplasm (Bell et al., 2000). Furthermore, the JNK1, a member of the mitogen-activated protein kinases, may play a key role in the pathogenesis of fat-induced insulin resistance (Hirosumi et al., 2002) and was suggested as a putative target of PKC-ε (Samuel et al., 2004); the latter authors also demonstrated that activation of both PKC-ε and JNK1 was prevented by reduction of hepatic lipid levels. These investigators recently provided further evidence suggesting that PKC-ε could directly inhibit insulin receptor kinase activity in vitro as well as in vivo (Samuel et al., 2007).

Although substantial evidence for the inhibition of insulin signaling has been provided, the mechanisms by which lipids, fatty acids, or their derivatives impair insulin resistance are not completely understood. Thus, besides free fatty acids or LCACoA, several factors may be involved in activation of insulin-signaling compromising protein kinases, such as cytokines, intracellular ceramide (Ruvolo, 2003), among others (McGarry, 2002; Roden, 2006; Mlinar et al., 2007). In this regard, cytokines such as IL-6, IL-1α, and TNF-α are of great importance and are elevated in obesity to exert paracrine effects in the development of insulin resistance (Hirosumi et al., 2002; Rotter et al., 2003; He et al., 2006; Andreozzi et al., 2007). This was deduced from studies with TNF-α knockout mice, which, unlike their wild-type counterparts, did not develop insulin resistance upon being fed a high-fat diet (Uysal et al., 1997) and from observations that anti-TNF-α antibodies ameliorated insulin resistance in skeletal muscle of maturing Sprague-Dawley rats (Borst et al., 2004). Indeed, TNF-α was demonstrated to interfere with insulin signaling in

both dependent and independent fashions from IRS-1 (de Luca and Olefsky, 2008). Induction of suppressor of cytokine signaling 3 has been connected not only to inhibition of insulin signaling by IL-6 but also to that by TNF-α (Ishizuka et al., 2007).

Insulin resistance may also mediated by other factors downstream of AKT/PI3K, which may be involved in impaired insulin signaling, such as structurally impaired cellular transport [e.g., of the insulin receptor (Inokuchi, 2006), GLUT4 transporter (Franck et al., 2007), and impaired lipid dynamics (i.e., defects or insufficiencies in lipid droplet (LD) associated proteins) (Kawanishi et al., 2000)]. Nonetheless, a direct link between intrahepatic lipid accumulation and insulin resistance has been challenged because of conflicting findings, generated in acyl-CoA:diacylglycerol acyltransferase 2 (DGAT) knockout mice. This enzyme catalyzes the last step in triacylglycerol synthesis, and although DGAT knockout mice displayed hepatic steatosis, they displayed no defects in glucose metabolism or insulin signaling (Monetti et al., 2007).

Nonetheless, metabolic overflow with lipids is a major determinant in primary NAFLD and thus leads to intrahepatic lipid accumulation. The intimate connection between insulin resistance and the development of NAFLD is characterized by an impairment of insulin sensitivity in the liver that results in failure to suppress the break down of glycogen and together with reduced peripheral glucose uptake leads to hyperglycemia. Hyperglycemia was suggested to be an additional trigger for lipogenesis in the liver and in hyperinsulinemia is associated with stimulation of de novo lipid synthesis in the liver.

In the state of mixed insulin resistance, the suppressive effects of insulin on gluconeogenesis are reduced, but insulin-stimulation may still mediate suppression of fatty acid oxidation in NAFLD livers (see Fig. 2B). In particular, phosphorylation of forkhead transcription factor Foxo1 by Akt is initiated via IRS-2 signaling and results in nuclear exclusion of the transcription factor and thus transcriptional repression of genes required for gluconeogenesis (e.g., phosphoenolpyruvate carboxykinase and glucose-6-phosphatase) (White, 1998; Nakae et al., 2001). In contrast, inhibition of hepatic β-oxidation through activity of, for instance, fatty acid synthetase (FAS) and stearoyl-CoA desaturase 1 is mediated by Foxa2, which is inactivated by phosphorylation by IRS-1 and IRS-2 signaling (Wolfrum et al., 2004) (Fig. 2A). In hyperinsulinemic and insulin-resistant mice, an impaired regulation of Foxo1 by insulin-stimulated IRS signaling was observed, whereas phosphorylation and nuclear exclusion of Foxa2 was maintained (Wolfrum et al., 2004). Thus, it was suggested that sustained inhibiting of fatty acid oxidation (FOXA2) via IRS-1 and IRS-2 during *mixed insulin resistance* contributes to intrahepatic lipid accumulation and development of hepatic steatosis (Fig. 2). In fact, transfection with an altered Foxa2 mutant, which is insensitive to insulin-

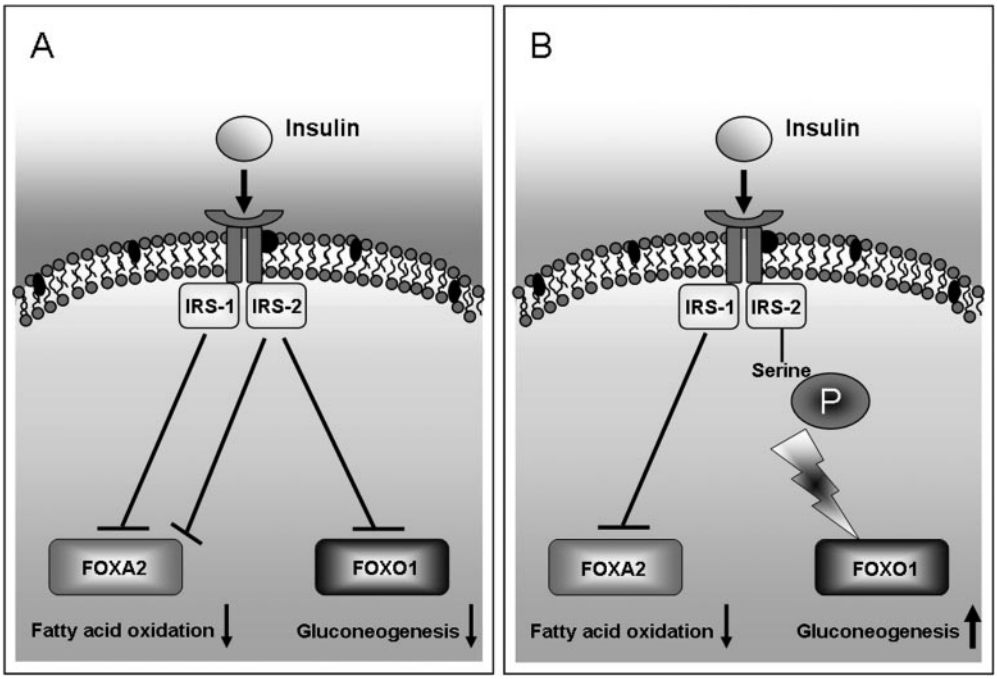

FIG. 2. Insulin signaling via insulin receptor substrates IRS-1 and IRS-2 in the presence of insulin resistance. Insulin secretion activates IRS-1 and IRS-2, and subsequent activation of AKT-dependent signal cascades inhibit FOXA2 and FOXO1 activity, leading to suppression of hepatic fatty acid oxidation and gluconeogenesis, respectively. A higher sensitivity to insulin and therefore a prolonged activation of FOXA2 during relative insulin deprivation compared with FOXO1 may be explained by a longer-lived activation of FOXA2 resulting from simultaneous stimulation of both IRS-1 and IRS-2. [Adapted from Montminy M and Koo SH (2004) Diabetes: outfoxing insulin resistance? *Nature* **432:**958–959 and from Puigserver P and Rodgers JT (2006) Foxa2, a novel transcriptional regulator of insulin sensitivity. *Nat Med* **12:**38–39. Copyright © 2004 and 2006. Reprinted by permission from Macmillan Publishers Ltd.].

dependent phosphorylation, into livers of diabetic mice, reversed hepatic steatosis and ameliorated insulin resistance (Wolfrum et al., 2004).

Furthermore, sustained suppression of FOXA2 in the presence of activated FOXO1 may contribute to an impaired hepatic lipid export via VLDL. Insulin inhibits hepatic VLDL secretion, as demonstrated in patients and animal models by interfering with maturation of VLDL, in an as-yet unknown way, which finally leads to a measurable increase in ApoB degradation (Patsch et al., 1986; Brown and Gibbons, 2001).

To further probe for the role of Foxa2 in the insulin-dependent control of VLDL secretion, Wolfrum and Stoffel studied the expression of Foxa2 and its coactivator PPARγ coactivator β (Pgc-1β) in livers of *ob*/*ob* mice. In essence, functional recovery of Foxa2 reduced hepatic triacylglycerol contents. This was achieved by induction of genes controlling mitochondrial β-oxidation and the gene coding for microsomal transfer protein—a key enzyme in VLDL synthesis—that led to increased ApoB-containing VLDL secretion (Wolfrum and Stoffel, 2006). Likewise, in the presence of insulin these processes were inhibited via a Foxa2-dependent mechanism (Wolfrum and Stoffel, 2006).

These data provide evidence for insulin-dependent suppression of FOXA2/Pgc-β to be a putative mechanism for hepatic lipid accumulation under conditions of insulin resistance. Forkhead transcription factors FOXO1 and FOXA2 are important switches between hepatic glucose and lipid metabolism, suggesting these proteins to be interesting targets for pharmaceutical interventions in steatosis, NASH, and insulin resistance.

B. Lipid Droplets—Metabolically Active Sites in Hepatic Steatosis?

The formation of lipid droplets (LD, also lipid bodies) is a physiological process and part of the specific function in various cell types, including adipocytes (energy reservoir), leukocytes (sites of storage and biosynthesis eicosanoids), and pneumocytes (production and metabolism of pulmonary surfactant), among others (Murphy, 2001; Wan et al., 2007). Although lipid storage in the form of LDs serves as long-term energy reservoir in adipocytes (Dugail and Hajduch, 2007), parenchymal hepatocytes provide short-term energy in form of glycogen. With regard to lipid metabolism, the liver uses lipids by uptake (neutral lipids, phospholipids, and free fatty acids from chylomicrons and lipoprotein particles) and fosters redistribution of lipids (in the form of lipoproteins) to peripheral storage in adipocytes or for its combustion, for instance, in skeletal muscle (Bradbury and Berk, 2004).

As discussed in section I, metabolic overload and production of ROS contribute to the development of NAFLD. However, the role of the hepatic lipid droplet and the switch from metabolic overload to inflammation has found little attention so far. Adipocytes have been investigated for lipid droplet formation and related dysfunctions, particularly in the context of obesity, insulin resistance, and diabetes type 2 (Guilherme et al., 2008). Based on studies with lipid-storing cells, common cellular programs can be deciphered that may translate to a better understanding of steatosis and NASH in conditions of metabolic overload with lipids.

It is noteworthy that adipocytes are specialized in the storage of triglycerides, but extensive overload with lipids results in hypertrophy of adipocytes and metabolic incompetence. Subsequently, macrophages infiltrate into adipose tissue, which coincides with the onset and development of inflammation. At early stages, an increase in caloric intake can be compensated by an increased expression of genes coding for triglyceride storage (Guilherme et al., 2008). Nonetheless, the size of adipocytes (possibly related to changes in the composition lipid droplet associated proteins) is an important determinant for insulin resistance, metabolic competence, and subsequent development of diabetes in patients with obesity (Puri et al., 2008; Straub et al., 2008, Franck et al., 2007)

It is noteworthy that lipid-overloaded adipocytes are incapable of appropriately disposing of incoming fat. It was proposed that elevated extracellular nonesterified free fatty acids activate macrophages via the TLR-4/NF-κB pathway (Shi et al., 2006). Mitochondrial dysfunction, ER, and other organelle stress are observed in adipocytes and hepatocytes upon excess supply with lipids (see section II.B.4 and de Ferranti and Mozaffarian, 2008). Indeed, hypertrophic adipocytes express monocyte-chemoattractant protein 1 (MCP-1), to facilitate activation and recruitment of the macrophage monocytic system (Kanda et al., 2006; Guilherme et al., 2008). In NAFLD, Kupffer cell infiltration is observed, as is elevated expression of MCP-1 in patients diagnosed with steatohepatitis (Bilzer et al., 2006). Macrophages secrete factors that interfere with the storage capacity of cells (Lagathu et al., 2006), such as cytokines (i.e., TNF-α and IL-6) that activate specific intracellular pathways in hepatocytes (e.g., proapoptotic signals and survival pathways, such as the NF-κB pathway (Tacke et al., 2008). In NAFLD and in obesity, TNF-α is elevated (Steinberg, 2007; Jarrar et al., 2008). It is noteworthy that TNF-α was found to impair insulin sensitivity and to repress TAG synthesis, esterification, and sequestration in adipocytes by down-regulation of PPARγ (for review, see Lacasa et al., 2007; Guilherme et al., 2008). The activity of nuclear receptor PPARγ is germane to lipid droplet formation (see section II.B for further detail). This transcription factor is an important regulator in lipid and carbohydrate metabolism and is involved in differentiation of preadipocytes to adipocytes by interaction with transcription factor CCAT-enhancer binding protein and the adipocyte differentiation and determination factor-1/SREBP-1 (Kallwitz et al., 2008). These characteristics made PPARγ an adequate target

for the treatment of the metabolic syndrome and is the subject of several reviews (Alberti, 2005; Staels, 2007; Bragt and Popeijus, 2008).

1. Lipid Droplet Formation. A role of PPARγ in lipid droplet formation was demonstrated in experiments were activation of PPARγ (i.e., by troglitazone) increased expression of lipid droplet associated proteins of the PAT family (perilipin, adipophilin/ADRP, and TIP47, S3–12, lipid storage droplet protein 5/oxidative tissues-enriched PAT protein (Arimura et al., 2004; Dalen et al., 2004; Motomura et al., 2006; Wolins et al., 2006). Lipid droplet-associated proteins adipophilin (ADRP) and perilipin are uniquely found in lipid droplets and are thought to be major effectors in the process of lipid droplet formation and lypolysis (Londos et al., 1999; Brasaemle et al., 2000; Miura et al., 2002). De novo expression of perilipin was recently confirmed in steatotic hepatocytes in which PAT expression correlated with the proportion of LD (Straub et al., 2008).

Overexpression of both adipophilin and perilipin is associated with an increase in TAG accumulation and lipid droplet formation and is physiologically stimulated by fatty acids (Gao and Serrero, 1999; Imamura et al., 2002; Fukushima et al., 2005; Dalen et al., 2006). Adipophilin is a free fatty acid transporter and is involved in the lipid transfer required for the formation of intracellular lipid droplets (Dalen et al., 2004; Wolins et al., 2005; Robenek et al., 2006; Wolins et al., 2006; Ducharme and Bickel, 2008).

It was proposed that lipid droplets evolve at the leaflets of the ER bilayer, where neutral lipids form discs by coalescence and subsequently enlarge to spheres and eventually bud from the ER into the cytoplasm to become surrounded by a phospholipid monolayer (Brown, 2001; Murphy, 2001). Freeze-fracture electron microscopy studies, however, demonstrated that in contrast to former notions, the lipid droplet is not situated within the ER membrane but lies external to it and is enclosed by both ER membranes, like an egg held by an egg cup (Robenek et al., 2006). Adipophilin is located within the ER membrane adjacent to the lipid droplet and has been implicated to orchestrate neutral lipid packing of the lipid droplet core (Robenek et al., 2006). In this regard, cytoplasmic lipid droplets in the liver have been determined to be in the range of 0.5 to 2.0 μm diameter (DiAugustine et al., 1973), and like the lipid droplets in adipocytes, they evolve at the endoplasmic reticulum (ER) membrane (Murphy, 2001), as detailed above.

2. PAT Proteins, Insulin Resistance, and Lipid Droplet Breakdown. Functional impairment of PAT family members, such as of perilipin, results in a dramatic increase in LD size and a decrease in LD number, as recently shown in an small interfering RNA approach with a murine leukemia cell line loaded with oleic acid (Bell et al., 2008).

In adipocytes, PAT family member perilipin is a key player in lipolysis, where it controls access and activity of HSL (Brasaemle et al., 2000). Perilipin A is located at the surface of intracellular lipid droplets and was proposed to sterically block access of hormone-sensitive lipase, thereby preventing hydrolysis of TAGs within adipose tissue (Blanchette-Mackie et al., 1995). Activation of lipolysis is triggered by phosphorylation of perilipin A at six serine residues by PKA and subsequent conformational changes that are thought to cause changes in the formation of lipid droplets to enhance their association with HSL and regulate its activity (Souza et al., 2002; Holm, 2003; Tansey et al., 2003; Moore et al., 2005; Miyoshi et al., 2006).

Recent findings, however, had questioned this model because it was shown that perilipin could also promote arrest of HSL (Miyoshi et al., 2006). Furthermore, perilipin induces translocation of PKA-phosphorylated HSL toward a subpopulation of small cytoplasmic lipid droplets, which are distinct from the major or central lipid storage (Moore et al., 2005), where it mediates association with lipid droplets and subsequent lipolysis (Sztalryd et al., 2003). During chronic inflammation, perilipin is down-regulated by TNF-α; this fosters lipolysis in the adipocyte and increases systemically available FFA to burden the liver with lipids (Guilherme et al., 2008).

Down-regulation of PAT proteins increased lipolysis by adipose triglyceride lipase and in insulin resistance (Bell et al., 2008). It was proposed that PAT proteins would act as surfactant at the LD surface to facilitate lipid droplet sequestration and processing into smaller units, thereby restricting access of lipases (Bell et al., 2008). Subdivision of LDs in response to lipolytic stimuli goes along with an amplification of the LD surface and may therefore provide larger contact surface for lipases and lipid-transporting proteins. This may well play a role in the micro- and macrovesicular steatosis of the liver, hepatic lipase activity being dependent on the lipid droplet surface. In liver microsome preparations, two lipases were identified (triacylglycerol hydrolase and arylacetamide deacetylase) that may contribute to hydrolysis of hepatic LDs (Lehner and Verger, 1997; Gibbons et al., 2000). This process was thought to take place at the ER membrane, where at the contact zone between LD and ER leaflet lipases release lipolytic products (Dolinsky et al., 2004). The role of LDs and associated proteins in an enhanced release of free fatty acids into the plasma during obesity is depicted in Fig. 3.

It is of considerable importance that allelic variants of the perilipin gene have been associated with BMI and the risk of developing obesity among women (Tai and Ordovas, 2007). Furthermore, a role for PAT proteins in the prevention of insulin resistance was deduced from experiments in which expression of genes coding for the lipid droplet-associated proteins perilipin and cell-inducing DFF45-like effector (CIDE) domain containing proteins CIDEA and FSP27 were positively correlated with the grade of insulin resistance in humans (Bell et al., 2008; Puri et al., 2008). Indeed, consistently elevated

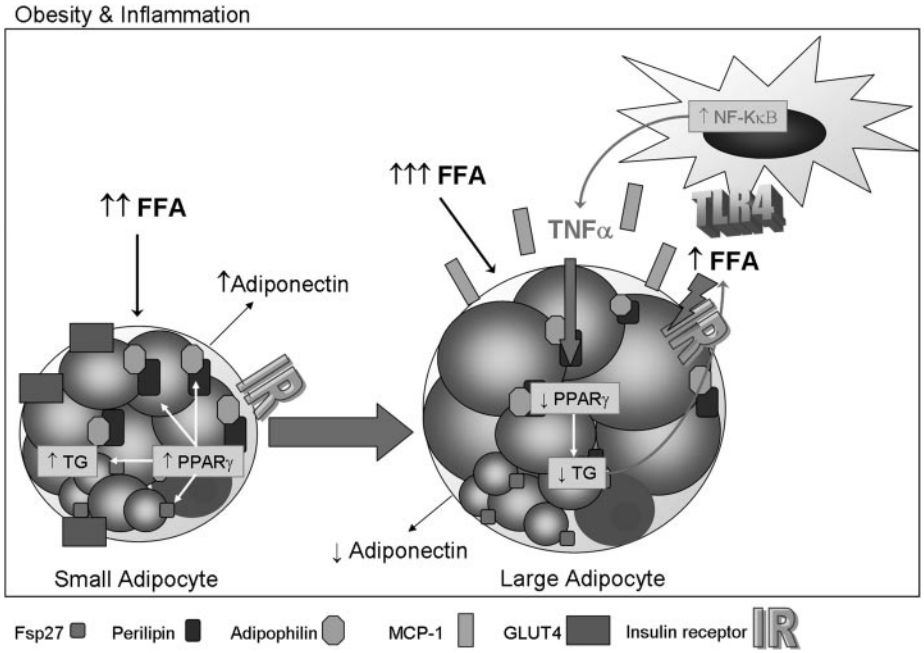

FIG. 3. Enlargement of lipid droplets during obesity are associated with an altered lipid and protein metabolism in lipid droplets. Whereas in small adipocytes, insulin binding to the insulin receptor (IR) results in suppression of lipolysis via perilipin, in large adipocytes, PPARγ-mediated expression of perilipin is reduced. This results in a storage defect, insufficient suppression of lipolysis, release of free fatty acids, and a defect in endocrine function (reduced adiponectin secretion). This effect is further triggered by TNF-α, which is released from macrophages. Finally, lipotoxic effect and cytokines may disturb insulin signaling.

expression of lipid droplet-associated proteins (LDAPs) is currently believed to be part of an adaptive strategy to improve lipid storage capacity in adipose tissue, whereas relative LDAP deficiency (with respect to the quantity of fat) was suggested to contribute to the metabolic and endocrine dysfunction in insulin resistance and T2DM (Bell et al., 2008; Puri et al., 2008).

This was supported by evidence from animal and clinical studies with PPARγ agonists, which enhanced LDAP expression, improved insulin resistance, and exerted beneficial effects in NASH, probably because of improved lipid storage in adipose tissue and subsequent facilitation of the redistribution from liver fat to the periphery (Miyazaki et al., 2002; Neuschwander-Tetri et al., 2003; Promrat et al., 2004; Kim et al., 2007).

3. PAT Proteins and PPARγ in Hepatic Steatosis. In adipose tissue and during obesity, PPARγ activity is diminished, as is the expression of PAT proteins. In contrast, activity of PPARγ is elevated in livers of patients with obesity, in NAFLD, and in animal models of NAFLD (Matsusue et al., 2003; Yu et al., 2003; Motomura et al., 2006; Westerbacka et al., 2007). Indeed, overexpression of liver-specific PPARγ2 induced steatosis, and this coincided with transcriptional activation of lipogenic genes, such as SREBP-1, FAS, acetyl-CoA carboxylase, and by activation of adipophilin (Schadinger et al., 2005). In obese mice, aberrant composition of PAT proteins have been reported (Bell et al., 2008), and similar findings were observed in steatotic human livers (Straub et al., 2008). There seems to be a correlation between grade of steatosis and expression of PAT proteins (Straub et al., 2008).

Induction of PAT proteins in the liver may result from hepatic lipid remodeling in states of insulin resistance, whereby PPARγ becomes activated by fatty acid ligands. Indeed, induced perilipin expression, was found in steatotic livers only and may serve as a backup system for limited lipid storage capacity of adipophilin in larger lipid droplets (Straub et al., 2008). Data from knockout experiments indicated that the different PAT family members were able to substitute for each other in their function to control the lipid storage in lipid droplets (Tansey et al., 2001; Larigauderie et al., 2006; Sztalryd et al., 2006). Together, these findings suggest, that similar to adipocytes, lipid droplet-associated proteins in hepatocytes play a role in lipid droplet formation and maintenance. Extensive lipid storage may contribute to failure of PAT protein function in the liver, resulting in impaired lipid metabolism and release of free fatty acids and activation of Kupffer cells by lipotoxic mechanisms. The hepatocyte fosters glycogen over lipid storage. This may be a reason for the comparably low capacity of hepatic lipid storage. Nonetheless, hepatic steatosis is reversible, particularly after weight loss and reduction of intrahepatic lipid contents (<200g of intrahepatic fat was estimated by magnetic resonance imaging and ^{1}H magnetic resonance spectroscopy) (Szczepaniak et al., 1999; Petersen et al., 2005). The fact that high-grade hepatic steatosis was found to be reversible within a few weeks after transplantation into human recipients

(Moon et al., 2006; McCormack et al., 2007) emphasizes the connection between hepatic steatosis and disorders in peripheral lipid storage. Consequently, improvement of peripheral lipid storage reduces lipid burdening of the liver and therefore reverses steatosis.

4. Lipid Droplets and Cell Signaling. Besides lipid storage, lipid droplets engage dynamically in the exchange of lipids and signaling molecules between various cellular organelles as well as the plasma membrane (Murphy, 2001). Through affiliation of LDs with lipid raft-associated proteins such as caveolins and flotillins, LDs contribute to intra- as well as intercellular communication (Martin et al., 2005; Liu et al., 2007a; Rajendran et al., 2007). Caveolins are the major proteins in specialized plasma membrane invaginations, the "caveolae." Caveolae may be functionally considered as specialized lipid rafts, which are dynamic components of the cell membrane characterized by their lipid content, as distinguished from the remaining membrane by its sterical order (Simons and Toomre, 2000; van Meer and Lisman, 2002) (Fig. 4).

In particular, caveolae are 50 to 100 nm in diameter and contain several receptors and transporters and are believed to play a central role in cholesterol homeostasis, sorting and transporting proteins, as well as in redirecting lipids to form lipid droplets (Severs, 1988; Fielding and Fielding, 1997; Simons and Ikonen, 1997; Anderson, 1998; Ostermeyer et al., 2001; Helms and Zurzolo, 2004). The 21-kDa protein caveolin is a integral membrane protein in caveolaes that by its cytoplasmic N-terminal domain associates with G-proteins, Src-like kinases, Ha-Ras, and endothelial nitric-oxide synthase (Li et al., 1995, 1996a; Song et al., 1996). A 20-amino acid region of the amino-terminal domain interacts with G-protein α subunits and Src-like kinases and negatively regulates their activity (Li et al., 1995, 1996b). Oligomers of caveolin with high molecular masses (~350 kDa) bind cholesterol (Murata et al., 1995) and glycosphingolipids (Li et al., 1996b), thereby acting as scaffolding proteins to orchestrate proteins and lipids in the formation of caveolae (Couet et al., 1997).

Furthermore, caveolae and other lipid rafts host receptor tyrosine kinases (RTKs), including TNF-α receptor, epidermal growth factor receptor, insulin receptor, PKC-α and have been linked to an internalization and signaling of these RTKs (Gustavsson et al., 1999; Legler et al., 2003; Puri et al., 2005; Kabayama et al., 2007). It is noteworthy that lipid rafts are thought to be critical for compartmentalization of insulin signaling; changes in lipid raft compositions have been suggested to be involved in insulin resistance of adipocytes (Yamashita et al., 2003; Kabayama et al., 2007). For instance, studies with caveolin-1 knockout mice had indicated that caveolae may be involved in stabilization of the insulin receptor protein in adipocytes. Furthermore, caveolin-1 knockout mice were found to be particularly sensitive to insulin resistance induced by a high-fat diet, which correlated with a 90% decrease in insulin receptor content of adipocytic caveolae (Cohen et al., 2003a,b). In contrast, mice lacking ganglioside GM3 displayed enhanced insulin sensitivity (Yamashita et al., 2003). A role for insulin receptor dissociation from caveolae in insulin resistance was further confirmed in 3T3-L1 adipocytes; TNF-α-induced loss of insulin sensitivity in adipocytes was accompanied by elimination of insulin receptors from caveolae paralleled with accumulation of the gan-

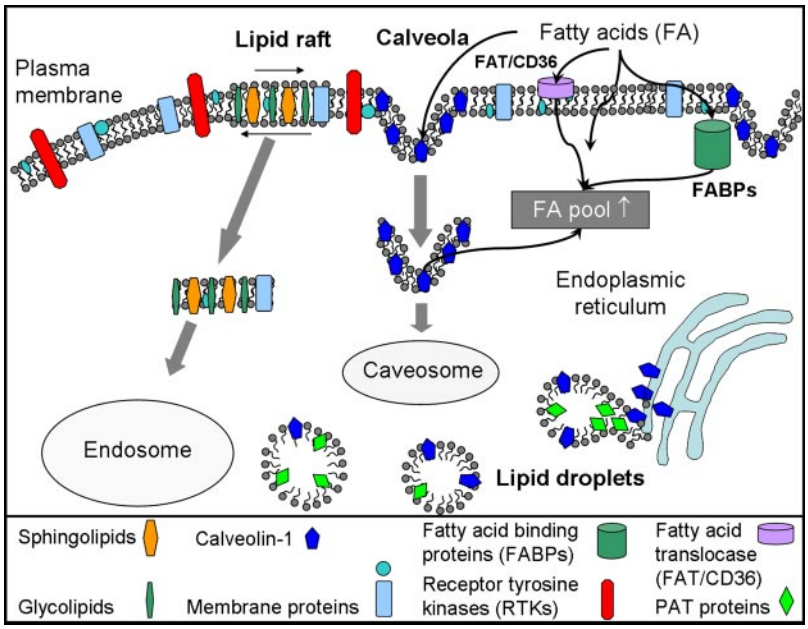

FIG. 4. Schematic overview of the lipid raft concept. Lipid rafts and caveolae are dynamic components of the phospholipid bilayer and are internalized into endosome and caveosome. Lipid droplets evolve at the endoplasmic reticulum, where lipid droplet-associated proteins caveolin-1 and PAT (perilipin, adipophilin, and TIP47) enable enclosure of triacylglycerols inside the lipid droplets.

glioside GM3 (Kabayama et al., 2005). Both caveolin-1 and ganglioside GM3 were shown to independently form complexes with insulin receptor; ganglioside GM3 enrichment in caveolae enhanced insulin receptor mobility (Kabayama et al., 2007). It was proposed that plasma membrane enrichment of ganglioside GM3 might be a pathological feature of insulin resistance that weakens the interaction between caveolin and insulin receptor to result in a displacement of insulin receptor from caveolae and subsequent prevention of insulin receptor signal transduction in adipocytes (Kabayama et al., 2005, 2007). The role for these processes in the pathogenesis of insulin resistance remains to be determined. Likewise, the ganglioside metabolism remains to be explored as putative target for future therapy of insulin resistance and such related disorders as NAFLD.

Altered lipid raft composition may provide a molecular rational for steatosis and steatohepatitis and may aggravate hepatic insulin resistance. Indeed, cholesterol depletion of adipocyte cultures disrupted caveolae and interfered with insulin signaling cascades and activation of downstream targets protein kinase B and MAPK extracellular signal-regulated kinase 1/2 (ERK1/2), to result in attenuation of insulin-dependent uptake of glucose (Parpal et al., 2001; Karlsson et al., 2004). Therefore, depletion of cholesterol affected insulin signaling downstream of IRS-1, which resulted in a loss of insulin-mediated phosphorylation of perilipin (Karlsson et al., 2004). Changes in phospholipid membrane compositions during steatosis and steatohepatitis may aggravate hepatic insulin resistance and lipid overload by altering caveolae-mediated functions, such as receptor tyrosine kinase signaling or uptake of long-chain fatty acids (LCFA) into liver cells (Pohl et al., 2002).

5. Lipid Droplets as a Connective Network. In contrast to former beliefs, lipid droplets are not necessarily independent of each other but are clustered and connected to each other, thereby constructing a continuous intracellular membrane system (membrane flow hypothesis) that enables exchange of lipids (Scow and Blanchette-Mackie, 1991; Binns et al., 2006). Physical interactions of lipid droplets with lipid-metabolizing organelles linked lipid droplets to activity of lipid metabolism (Martin et al., 2005). It is noteworthy that recent evidence has suggested that lipid droplets might also be involved in the regulation of fatty acid oxidation itself (Binns et al., 2006). Adipophilin knockdown, for instance, was associated with a decrease of lipogenic genes in a rodent model of NAFLD (Imai et al., 2007). Furthermore, the close proximity of LDs to mitochondria and peroxisomes suggests the existence of a mechanism for lipid oxidation via substrate supply (Blanchette-Mackie et al., 1995; Cohen et al., 2004). Presence of mitochondrial, ER-related, and peroxisomal proteins in lipid droplets substantiates morphologic observations of lipid droplets interacting with peroxisomes and possibly mitochondria and ER (Blanchette-Mackie et al., 1995; Cohen et al., 2004; Binns et al., 2006). Binns et al. (2006) demonstrated that lipid droplets in *Saccharomyces cerevisiae* occasionally contain extensions of peroxisomes, which they termed "pexopodia." Occurrence of these contact zones is closely connected to nutritional state of the cell and may enable substrate supply and distribution of fatty acids to lipid-metabolizing organelles. So far, both mitochondrial and peroxisomal defects provide sufficient rationale for the pathogenesis of hepatic steatosis (Reddy and Hashimoto, 2001; Zhang et al., 2007). Thus, defects in lipid droplet interactions with lipid-metabolizing organelles may promote lipid storage. Little is known about a possible role of lipid droplets in the protection of cells by disposing toxic lipids (Yamaguchi et al., 2007), such as nonesterified fatty acids (Cnop et al., 2001; Mishra and Simonson, 2005), lipid peroxidation products (e.g., oxidized phosphatidylcholine) (Ikura et al., 2006), or excessive lipid mediators of intracellular signaling [e.g., prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids, and epoxyeicosatetraenoic acids (Wolins et al., 2006)].

C. The Role of Fatty Acids in Steatosis

Reduced intracellular availability of polyunsaturated fatty acids (PUFAs) and altered lipid composition in phospholipid bilayers of steatotic livers have been proposed to contribute to exacerbation of steatosis by enhancing lipogenic lipid metabolism and production of inflammatory molecules. PUFAs have been attributed to anti-inflammatory and antilipogenic effects serving as a backup system to capture radicals but also by acting as ligands for nuclear transcription factors.

1. Fatty Acids as Regulators of Lipogenic Gene Expression. PUFAs have been demonstrated to decrease expression of prolipogenic nuclear receptors, such as SREBP-1 and ChREBP, but also to decrease DNA binding of transcription factor NF-Y, which regulates expression of the FAS gene (Ou et al., 2001; Yoshikawa et al., 2002; Dentin et al., 2005, Swagell et al., 2007; Teran-Garcia et al., 2007). In the case of transcription factor SREBP, polyunsaturated free fatty acids reduce intracellular levels of the active transcription factor, thereby decreasing gene expression mediated by sterol regulatory elements (SREs) for up to 20 to 75% in a dose-dependent manner (Worgall et al., 1998). This effect was also observed in vivo, where treatment with PUFAs decreased mRNA stability of hepatic SREBP-1 and SREBP-2 and enhanced mRNA decay of ChREBP in rodents fed diets enriched with fish oil or linoleate (C18:2), eicosapentanoic acid (C20:5), or docosahexaenoic acid (C22:6), respectively (Xu et al., 2002; Dentin et al., 2005).

So far, the ways in which PUFAs interfere with nuclear receptors are not completely understood but were associated with transcriptional and post-transcriptional regulatory mechanisms (Deckelbaum et al., 2006). In the case of ChREBP, PUFAs were found to inhibit ac-

tivity of the transcription factor by preventing ChREBP translocation from the cytosol to the nucleus (Dentin et al., 2005).

Molecular principles underlying mechanisms of PUFA-mediated reduction of lipogenic transcription factors have been highlighted in studies with the SREBP transcription factors. As such, it was proposed that PUFAs could interfere with active levels of these transcription factors by interacting with their intracellular transport. SREBPs are transcription factors that are post-transcriptionally regulated. The premature form of SREBP is linked to the ER and is transported to the Golgi apparatus, where it dissociates from a complex with SREBP cleavage-activating protein and undergoes proteolytic cleavage to be relieved in its transcriptional active form (Deckelbaum et al., 2006). Upon translocation into the nucleus, SREBPs activate *cis*-acting elements in the promotors of genes of cholesterol and fatty acid synthesis, the sterol regulatory elements (SREs). The lipogenic activity of SREBPs is physiologically limited by a negative feedback loop triggered by cholesterol, which inhibits the proteolytic cleavage of SREBs in the Golgi apparatus, thereby reducing the release of transcriptional active SREBPs (Deckelbaum et al., 2006). PUFAs were proposed to retain the premature form of SREBPs linked to the ER and decelerate their transport to the Golgi apparatus by increasing translocation of plasma membrane cholesterol to intracellular compartments, such as the ER.

This effect on cholesterol transport was explained by activation of plasma membrane-associated sphingomyelinases by PUFAs and subsequent sphingomyelin hydrolysis (Robinson et al., 1997). Because cholesterol has a high affinity to sphingomyelin, reduction of plasma membrane sphingomyelin promotes transport of cholesterol from the plasma membrane to cholesterol-poor intracellular compartments (e.g., ER) (Subbaiah et al., 2008). Another independent mechanism has been related to the generation of ceramide through PUFA-mediated activation of sphingomyelin hydrolysis. It was demonstrated that increasing intracellular ceramide levels by addition of exogenous sphingomyelinase, ceramide analogs, or inhibition of ceramide breakdown sufficiently decreased SRE-mediate gene expression in reporter assays independent of changes in cholesterol transport dynamics (Worgall et al., 2002; Subbaiah et al., 2008).

Finally, PUFAs were found to lower activity of lipogenic transcription factors by interfering with the crosstalk of nuclear receptors. Such PUFAs were reported to competitively inhibit activation of SREBP by binding to liver X receptor (LXR), thereby preventing LXR/retinoid X receptor (RXR) heterodimer to bind to the LXR response elements in the SREBP-1c promoter (Ou et al., 2001; Yoshikawa et al., 2002). This effect was possibly mediated by PUFA-induced activation of PPARα, which inhibited formation of LXR/RXR heterodimers (Yoshikawa et al., 2003).

2. Polyunsaturated Fatty Acids in Nonalcoholic Hepatic Steatosis and Steatohepatitis. Changes in the lipid body compositions have been reported for patients with insulin-resistant NAFLD and have been characterized by 1) reduction in long-chain fatty acids, 2) increased *n*-6/*n*-3 PUFA ratios in liver and adipose tissue, 3) increased 18:1 *n*-9 *trans* levels in adipose tissue, 4) and elevated markers of hepatic lipid peroxidation and protein oxidation (Araya et al., 2004; Konishi et al., 2006).

Depletion of *n*-3 PUFAs in hepatic steatosis may result from dietary causes or inhibition of hepatic desaturases as a result of excessive exposure to ROS and subsequent lipid peroxidation (Das, 2004). Essential fatty acids, such as linoleic acid (18:2, *n*-6) and linolenic acid (18:3, *n*-3) are precursors of *n*-3 and *n*-6 PUFAs and therefore must be obtained from diets. Activity of Δ^5 and Δ^6 desaturases and elongases subsequently convert these fatty acids into their *n*-3 and *n*-6 metabolites. The desaturases, which are key enzymes in biosynthesis of *n*-3 and *n*-6 PUFAs are inhibited in patients with obesity and can be blocked by alcohol and elevated levels of *trans*-octadecenoic acid (18:1, *n*-9, *trans*) (Mahfouz et al., 1984; Nakamura et al., 1994; Medeiros et al., 1995; Larqué et al., 2000; Das, 2005).

The depletion of *n*-3 PUFAs in phospholipid bilayers of the liver such as by dietary inhibition of desaturases or excessive oxidative damage was proposed to be a central event in the development of hepatic steatosis by altering the hepatic lipid metabolism (Videla et al., 2004). Loss of PUFA-mediated activation of nuclear transcription factor PPARα and loss of their suppressive effect on lipogenic transcription factors SREBPs was attributed to reduced fatty acid oxidation, VLDL secretion, and reduced suppression of cholesterol and fatty acid synthesis, respectively. Thus depletion of PUFA is seen as a lipogenic factor that may enhance hepatic lipid accumulation by shifting the hepatic lipid metabolism from lipid oxidation to triglyceride storage (Matsuzaka et al., 2002; Yoshikawa et al., 2002).

Furthermore, a decrease in hepatic *n*-3 PUFAs, which are more effective activators of PPARα signaling than *n*-6 PUFAs, was proposed to be causally involved in a loss of PPARα-related anti-inflammatory and antilipogenic effects (Carlsson et al., 2001; Delerive et al., 2001; Lindén et al., 2002). Changes in the hepatic lipid composition in patients with NASH and animal models of NASH have previously highlighted the importance of the *n*-6/*n*-3 ratio of long-chain PUFAs for the progression from steatosis to steatohepatitis (Araya et al., 2004; Li et al., 2006). In turn, increased availability of *n*-6 PUFAs (e.g., arachidonic acid) seems to result in enhanced production of proinflammatory lipid mediators in phospholipid membranes of steatotic livers, which has been suggested to contribute to progression of steatosis

via activation of Kupffer cells and subsequent increase in ROS exposure of hepatocytes (Videla et al., 2004). In fact, cyclooxygenase (COX)-dependent generation of prostaglandins (e.g., PGE_3) from *n*-3 PUFAs was found to exert less inflammatory potential compared with *n*-6 PUFA-derived PGs (Bagga et al., 2003). Thus, successive depletion of *n*-3 PUFAs by multiple systemic and local factors may represent a putative pathway for the frontier of steatosis being crossed toward steatohepatitis.

3. Therapeutic Effects of Polyunsaturated Fatty Acids in Nonalcoholic Hepatic Steatosis and Steatohepatitis. Studies with dietary supplementation of *n*-3 PUFAs in *ob*/*ob* mice demonstrated their potency to ameliorate hepatomegaly and steatosis (Sekiya et al., 2003; Levy et al., 2004; McCullough, 2006a). The positive effects of polyunsaturated fatty acids (PUFAs) in vivo have been mainly attributed to their ability to redirect glucose into glycogen storage and fatty acids from triglyceride storage into lipid oxidation (Videla et al., 2004). This "repartitioning" contributed to their rather beneficial effects, such as reduction of blood serum levels of VLDL, triacylglycerols, and cholesterol and decrease of insulin resistance (de Lorgeril and Salen, 2006). Other positive treatment effect of *n*-3 PUFAs has been related to their interference with insulinotropic effects of saturated fatty acids, lowering insulin-dependent stimulation of lipogenic genes in the liver (Holness et al., 2004). PUFA-mediated activation of PPARα was demonstrated to antagonize detrimental effects on pancreatic β-cells in vitro, being able to rescue β-cell function (Holness et al., 2007) and advantageous effects of PUFA supplementation in steatosis were suggested to result from an improvement of peripheral insulin resistance, which was demonstrated in vitro but not supported by findings in vivo (Fickova et al., 1998; Ryan et al., 2000; Holness et al., 2004). Finally, another putative protection mechanism of unsaturated fatty acids in steatosis and steatohepatitis was attributed to their antioxidant effects, serving as a cellular reservoir for undue lipid peroxidation (Davis et al., 2006; Oliveira et al., 2006). Quite contrary to this assumption, it was recently demonstrated that feeding mice a *n*-3 PUFA-enriched fish oil diet in the methionine- and choline-deficient (MCD) model of steatohepatitis, led to robust activation of hepatic PPARα and subsequently reduced hepatic lipid accumulation but was also associated with marked hepatic accumulation of lipid peroxides, compared with control mice or mice fed an olive oil-enriched diet (Larter et al., 2008a). Although *n*-3 PUFAs may sufficiently suppress hepatic de novo lipogenesis, high levels of hepatic lipoperoxides may have aggravated steatohepatitis by lipotoxic hepatocellular injury and inflammatory recruitment in this model. As of today, the therapeutic benefit of a dietary supplementation with *n*-3 PUFAs in patients diagnosed with nonalcoholic fatty liver disease remains to be confirmed in clinical trials.

4. Roles for Saturated and Monounsaturated Fatty Acids in Nonalcoholic Hepatic Steatosis and Steatohepatitis. Although unsaturated fatty acids have positive effects on hepatic lipid metabolism and hepatic cells in steatosis, unsaturated fatty acids such as palmitate negatively affect cell survival by inducing lipoapoptosis and chemokine secretion (Unger and Orci, 2002; Malhi et al., 2006; Weinberg, 2006; Joshi-Barve et al., 2007). Exposure to palmitic acid was shown to activate NF-κB and activator protein-1, induced dose- and time-dependently IL-8 expression in HepG2 cells as well as in cultures of primary human and rat hepatocyte cultures (Joshi-Barve et al., 2007). Likewise, treatment of primary rat hepatocyte cultures with stearic acid (18:0) and oleic acid (18:1) for 24 h significantly increased IL-10 levels in cell culture media, whereas linoleic acid (18:2) and linolenic acid (18:3) had no such effect (Nishitani et al., 2007).

It is noteworthy that observations that monounsaturated FAs also had protective effects in hepatic steatosis, although they had a stimulating effect on triglyceride synthesis, led to introduction of an interesting hypothesis stating that monounsaturated FAs may prevent palmitate-induced lipoapoptosis by channeling excess saturated FAs toward triglyceride synthesis and lipid storage away from activation of lipotoxic cell death via metabolism of palmitate to ceramide classes (Listenberger et al., 2003; Damelin et al., 2007). Therefore, intracellular triglyceride storage in the liver may protect, at least in part, from oxidative stress or lipotoxins. This was also suggested in an animal model, where interruption of triglyceride synthesis by knockdown of diacylglycerol acyltransferase, the final step in TAG biosynthesis improved hepatic steatosis in MCD-fed mice but caused exacerbation of liver injury. This aggravation of liver injury was probably caused by increasing intracellular levels of free fatty acids, oxidative stress, inflammation, and fibrosis (Yamaguchi et al., 2007). The role of intracellular lipid accumulation as cellular protection mechanism for oxidative stress was further supported in an in vitro model of fat-loaded (palmitic or oleic acid) HepG2 spheroids, which, when challenged with pro-oxidants, were found to display lower levels of cytotoxicity and increased antioxidant activity than nonsteatotic controls (Damelin et al., 2007).

It was demonstrated that lipid overload with fatty acids independent of their saturation grade results in hepatic lipid accumulation in vitro. Mice fed a MCD-diet supplemented with 20% saturated or unsaturated fatty acids developed hepatic steatosis with signs of lobular inflammation irrespective of their diet (Larter et al., 2008b) and despite a reduction of hepatic SREBP-1 and substantial suppression of the triglyceride synthesis pathways. Whether depletion of PUFAs plays a central role in the development of steatosis upon overnutrition remains elusive.

D. Molecular Causes Resulting in Steatohepatitis

1. Nuclear Receptors and Transcription Factors in Steatosis/Nonalcoholic Hepatic Steatosis and Steatohepatitis. Fatty acids are known to be ligands for nuclear transcription factors, such as PPARα and hepatic nuclear factors (HNFs), and have been regarded as metabolic regulators of fatty acid oxidation.

The major transcription factors involved in nutritional control of the lipid metabolism are SREBP-1, PPARγ, ChREBP, lipogenic liver X receptor (LXR), forkhead box 01 (Foxo1), Foxa2, and PPARα, which controls fatty acid degradation, but also apolipoprotein AI regulatory protein-1, EAR-2, EAR-3, and HNF-4, which are all members of the steroid receptor superfamily and are involved in the control of lipoprotein metabolism (Ladias et al., 1992; Ide et al., 2003; Canbay et al., 2007; Cha and Repa, 2007). As a result of interactions with cellular lipids and dietary fatty acids, these nuclear transcription factors (TFs) control gene expression of genes coding for glucose and lipid metabolism via a complex TF network (Jump, 2002). Key players in hepatic lipid accumulation are PPARs. For instance, liver-specific expression of PPARα is activated by binding to exogenous and endogenous ligands, such as xenobiotics (e.g., fibrates), eicosanoids, and fatty acids (Mehendale, 2000; Motojima and Hirai, 2006). These characteristics have made PPARα an efficient intracellular lipid sensor (Motojima and Hirai, 2006). In addition to a ligand-dependent transactivating domain, PPARα receptors contain a NH_2-terminal ligand-independent transactivating domain and a DNA-binding domain with two zinc finger motifs (Xu et al., 2001). Upon heterodimerization with RXR, PPARα binds to peroxisome proliferator responsive elements and augments expression of genes coding for enzymes of mitochondrial and peroxisomal β-oxidation (Kane et al., 2006). Important genes in the oxidation of fatty acids in humans containing at least one consensus sequence for PPARα are mitochondrial carnitine palmitoyl transferase-I and -II, peroxisomal acyl-CoA oxidase, and LCACoA synthetase, which is required for activation of fatty acids to LCACoA (Fatehi-Hassanabad and Chan, 2005). Other target genes involved in the lipid metabolism of PPARα are mitochondrial HMG-CoA synthase (ketogenesis), cytochrome P450 enzymes (fatty acid and cholesterol metabolism), phospholipid transfer protein [high-density lipoprotein (HDL) metabolism] and apolipoprotein-AI and -AII (plasma HDL metabolism) (Fatehi-Hassanabad and Chan, 2005). It is noteworthy that PPARα knockout ($-/-$) mice display severe hepatic steatosis upon fasting as a result of failure to up-regulate the fatty acid oxidation system (Ip et al., 2003) Proper activation of PPARα is required to enhance hepatic lipid turnover to enable sufficient clearance of lipids from the liver, preventing lipid accumulation and peroxidation in murine NASH models system (Ip et al., 2003; Harano et al., 2006). Activity of PPARα is enhanced by phosphorylation of serine residues S12 and S21 upon insulin treatment and impaired by high-fat diets, alcohol, and inflammation (Juge-Aubry et al., 1999; Galli et al., 2001; Nanji et al., 2004; Alwayn et al., 2006; Svegliati-Baroni et al., 2006).

In contrast, pharmacological stimulation of PPARα by ligands (e.g., by fibrates and *n*-3 PUFAs) was effective in preventing intracellular lipid accumulation and attenuated steatosis in an animal model of nonalcoholic fatty liver disease (Reddy and Hashimoto, 2001; Akbiyik et al., 2004; Harano et al., 2006; Svegliati-Baroni et al., 2006). Besides its direct effect on lipid oxidation, PPARα has been suggested to control fatty acid influx into mitochondria and rates of β-oxidation via modulation of malonyl CoA levels. PPARα induces malonyl CoA decarboxylase, which degrades malonyl-CoA and is able to control CPT-1 activity and substrate supply for β-oxidation (Lee et al., 2004). However, recent findings with rat hepatoma cells indicated that transcription factors other than PPARα may be responsible for the induction of CPT-1. Although overexpression of a mutated transcriptional inactive PPARα receptor in rat hepatoma cells was shown to inhibit fibrate-mediated CPT-1 gene expression, no effect on LCFA-induced expression of CPT-1 was observed (Le May et al., 2005). Furthermore, the region responsible for the stimulatory effect of LCFA on CPT-1 was located in the first intron of the *CPT-1* gene, which contained no consensus sequence for binding of PPARα, -β, -γ, HNF4, or RXR (DR1) as well as for LXR (DR4), as determined by bioinformatic analysis (Louet et al., 2001). Finally, CPT-1 activity was demonstrated to be regulated by a nontranscriptional covalent modification, which may be particularly important for short-term regulation in response to acute intracellular signaling (Kerner et al., 2004).

Reduced PPARα activity may contribute to an imbalance of inflammatory signals, which was related to a loss of PPARα-mediated anti-inflammatory effects, such as induction of IκBα gene expression and reduced NF-κB DNA-binding affinity. It has been furthermore proposed that PPARα inhibits translocation of NF-κB to the nucleus by interacting with p65 (Delerive et al., 2001). Thus its role in the negative regulation of inflammation may be the second important effect of PPARα in hepatic steatosis. Through inhibition of NF-κB, PPARα prevents induction of pro-inflammatory cytokine and enzyme expression, such as TNF-α and COX II (Yu et al., 2006). In fact, activation of PPARα was recently found to protect from obesity-induced inflammation in murine models by both down-regulation of pro-inflammatory chemokines and up-regulation of anti-inflammatory factors, such as IL-1 (Stienstra et al., 2007a,b). Taken collectively, a diminished or impaired physiological activation of PPARα may dramatically reduce the liver's ability to accomplish lipid catabolism and thereby may be causally involved in the development of steatosis (Reddy, 2001).

Furthermore, activation of transcription factor PPARγ was linked to prosteatotic effects (Boelsterli and Bedoucha, 2002). PPARγ activates a number of genes that lead to enhanced uptake of glucose and lipids, increase glucose oxidation, and decrease free fatty acid concentration and insulin resistance (Way et al., 2001; Dumasia et al., 2005).

The latter is believed to be mainly influenced by PPARγ-mediated expression of adiponectin receptors and negative regulation of TNF-α, leptin, and pro-inflammatory cytokines produced by adipocytes (Hotamisligil et al., 1993; Kallen and Lazar, 1996; Jiang et al., 1998; Lehrke and Lazar, 2005; Ding et al., 2007). The anti-inflammatory effects of PPARγ result from interference with proinflammatory transcription factors, as demonstrated for NF-κB, which is inhibited by physical interaction of PPARγ and p65 and p50 subunits, thereby preventing degradation of cytoplasmic inhibitor IKK-β and subsequent transactivation of NF-κB (Chung et al., 2000). The exact interactions between PPARγ and NF-κB, however, are not yet dissected but may involve modulation of the IKK-β and also MAPK signaling pathway (Misra et al., 2002).

A cross-road between these two pathways has been observed before apoptosis in colon cancer cells, in which inhibition of PPARγ activity by Erk1/2-dependent phosphorylation was shown to inhibit NF-κB by increasing the physical interaction of PPARγ with p65 (Chen et al., 2003). Activity of PPARγ decreases its transcriptional activity; this was demonstrated for JNK and ERK2 after stimulation with EGF (Ser82 and Ser84 of PPARγ) (Adams et al., 1997; Camp and Tafuri, 1997) and p42/p44 MAP kinase (at Ser112) in response to insulin treatment (Hu et al., 1996).

Furthermore, PPARγ represses the *inducible nitric-oxide synthase* (*iNOS*) gene by inhibiting DNA binding of activator protein-1, signal transducer and activator of transcription-1, and NF-κB by targeting cAMP response element-binding protein (Li et al., 2000). Although adipocytes display high expression levels of the PPARγ2 isoform, which is required for adipocyte differentiation, the nonadipocyte isoform PPARγ1 is expressed only at very low levels in the healthy liver (Vidal-Puig et al., 1997). However, PPARγ is elevated in the livers of animals that develop fatty livers (Schadinger et al., 2005; Zhang et al., 2006). Fatty acids, such as γ-linolenic acid, eicosatrienoic acid, eicosapentaenoic acid acid, dihomo-γ-linolenic acid, and arachidonic acid, as well as their eicosanoid metabolites (e.g., 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2) and thiazolidinediones are ligands to PPARγ (Forman et al., 1995; Xu et al., 1999). Although PPARα and -γ have common ligands, the affinity of PPARγ for unsaturated fatty acids is remarkably low and even lower for saturated fatty acids, suggesting that PPARγ is activated only under conditions of lipid burdening (Xu et al., 1999).

Intrahepatic lipid levels are increased by elevated expression of PPARγ target genes involved in the lipid metabolism, such as lipoprotein lipase, FAS, and acetyl-CoA synthetase (Bogacka et al., 2004), but also by lipid droplet-associated and -inducing proteins ADRP and oxidative tissues-enriched PAT protein (Schadinger et al., 2005; Wolins et al., 2006). It is noteworthy that findings based on microarray analysis in steatotic livers of mice indicated that up-regulation of PPARγ occurs as protective response to suppress genes coding for pro-inflammatory cytokines, such as SAA, chemokine (C-X-C motif) ligand 10 (CXL10/IP10) (Yu et al., 2003). Thus, activation of PPARγ may be part of an adaptive response to lipid-induced pro-inflammatory stimuli.

In addition, the nuclear receptors and transcription factor SREBPs, which are members of the basic helix-loop-helix leucine zipper family, are key regulators of nutritional induction of lipogenic enzymes (Duplus and Forest, 2002). SREBP-1(−/−) mice being fed a carbohydrate diet display severely impaired induction of hepatic genes coding for fatty acid synthesis (e.g., acetyl-CoA carboxylase, FAS, and stearoyl-CoA desaturase) and display complete abrogation of gene transcription of lipogenic enzymes such as glycerol-3-phosphate acyltransferase, ATP citrate lyase, malic enzyme, and glucose-6-phosphate dehydrogenase (Shimano et al., 1999). Overexpression of SREBP-1a in adipose tissue of mice induced adipocyte hypertrophy, led to an increased fatty acid release, and led to development of fatty liver (Horton et al., 2003b). Vice versa, inhibition of SREBPs by dietary supplementation of PUFAs enhanced lipid oxidation and reduced lipogenesis (Xu et al., 2002; Yahagi et al., 2002; Sekiya et al., 2003). In addition to the effects of PUFAs on the processing of inactive SREBP precursor in the ER as well as on SREBP mRNA stability (described in section II.C.2), activation of AMP-activated protein kinase (AMPK) was found to decrease SREBP expression (Zhou et al., 2001). The underlying mechanism of AMPK-mediated inhibition of SREBP is not fully understood, but may include an enhanced mRNA instability as well as AMPK-mediated activation of Insig-1, i.e., a protein located in the ER that is responsible for the sterol-dependent transport and release of SREBP from the ER (Zhou et al., 2001). Upon activation of Insig-1 the SREBP/SREBP cleavage-activating protein complex is retained in the ER, thereby preventing SREBP-mediated effects on expression of lipogenic genes (Engelking et al., 2004; Roth et al., 2008). Induction of Insig-1 gene expression was recently linked to the activity of transcription factors constitutive active/androstane receptor (CAR) and PXR (pregnane X receptor) (Roth et al., 2008). The detection of a DR-4 binding site for CAR and PXR in the upstream promoter region of the Insig-1 gene could provide a mechanistic explanation for an inhibition of lipogenesis observed under treatment with 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene and phenobarbital ligands of CAR and PXR, respectively (Hall et al., 1990; Locker et al., 2003; Roth et al., 2008). The exact pathways, including Insig-1 induction by PXR and CAR as

well as its activation via AMPK, remain to be investigated and may lead to novel therapeutic approaches.

Clinical studies had supported a role for SREBP-1 in hepatic steatosis. In insulin-resistant lipodystrophic HIV infection, hepatic steatosis was found to be associated with overexpression of SREBP-1, as evidenced by liver biopsies (Lemoine et al., 2006). Furthermore, in a study of 40 patients with obesity, a positive correlation between increased prevalence of obesity, hypertriglyceridemia, and diabetes type 2 in patients displaying one of six different investigated single nucleotide gene polymorphisms of the SREBP-1 gene was observed (Eberlé et al., 2004). Gene polymorphisms of SREBP-1 may thus predispose to metabolically induced steatosis.

In this regard, the LXR is another important regulator of cholesterol homeostasis and of bile acid metabolism, which, upon activation, induces elevated hepatic fatty acid synthesis, increases secretion of triglyceride-rich very-low-density lipoprotein (VLDL), and fosters development of hepatic steatosis in mice (Schadinger et al., 2005; Cha and Repa, 2007), an effect that was explained by regulation of nuclear transcription factors SREBP-1c and ChREBP via LXR (Cha and Repa, 2007).

It is noteworthy that LXR is one of the potential candidates for the switch between steatosis and necrosis. In particular, activation of LXR was found to protect against hepatic injury in an endotoxemia model (Wang et al., 2006c). LXR was shown to attenuate LPS-induced release of TNF-α and PGE_2 in a dose-dependent fashion, suggesting that LXR activation may protect against liver injury by suppressing Kupffer cell activation (Wang et al., 2006). LXR is activated via PPARγ (Chawla et al., 2001), which attenuates liver fibrosis. From in vitro experiments, it could be demonstrated that reduced activity of PPARγ is necessary to evoke transdifferentiation of hepatic stellate cells into myofibroblastic-type cells (Marra et al., 2000; Tsukamoto, 2005). If LXR is indeed the switch that turns the steatotic phenotype into fibrosis, it is likely that steatosis and fibrosis are opposing fates in which liver injury may progress (Tsukamoto, 2005). More experimental work will be required to elucidate the role of LXR in regulation of steatosis and fibrosis.

Transcriptional regulation of lipogenic genes in response to glucose is performed by transcription factor ChREBP. This transcription factor is overexpressed in livers of *ob*/*ob* mice and liver-specific inhibition of ChREBP by introduction of short hairpin RNAs was found to improve hepatic steatosis in these animals (Dentin et al., 2006). Coordinate activation of TFs, such as SREBP-1 and ChREBP, is an important step in the proper functioning of lipogenic and lipolytic pathways.

A further transcription factor of considerable importance is HNF4α (designated NR2A1), a DNA-binding zinc finger protein that belongs to the hepatocyte nuclear factor subfamily. HNF4α controls the expression of genes involved in glucose, lipid, and xenobiotic metabolism and is involved in the regulation of developmental processes of the liver as well as in later events of hepatocellular differentiation (Schrem et al., 2002). Target genes of HNF4α are the nuclear transcription factors HNF1α, PXR, and CAR and genes such as *aldolase B*, *apolipoproteins*, *L-fatty acid binding protein*, *Cyp7a1*, the rate-limiting step in bile acid biosynthesis, as well as other genes involved in xenobiotic and lipid as well as carbohydrate metabolism (Fang et al., 2007; Onica et al., 2008). HNF4α is post-transcriptionally regulated, and specificity and affinity to DNA is either lowered or enhanced by phosphorylation at serine, threonine, and tyrosine residues via respective kinases (e.g., p38 kinase, PKA) (Schrem et al., 2002; Xu et al., 2007). Fatty-acyl thioesters [e.g., (C14:0)-CoA] are agonistic ligands of HNF4α that increase the binding of the HNF4α dimers and enhance their DNA binding (Wu et al., 1997; Schrem et al., 2002). In contrast, long-chain polyunsaturated and saturated fatty acyl-CoAs lower the binding dimerization of HNF4α and DNA-binding affinity of HNF4α dimers to their cognate enhancer element, thereby lowering HNF4α-mediated gene transcription (Hertz et al., 1998).

Mutations in HNF4α result in loss of gene function and have been linked to the development of maturity-onset diabetes of the young type 1 (MODY1) and non–insulin-dependent diabetes (Yamagata et al., 1996; Ryffel, 2001). Besides an impaired function of pancreatic α-cells along with an abnormal glucagon secretion, patients with MODY1 display lower serum concentrations of apolipoproteins apoCIII, apoAII, and apoB (Lehto et al., 1999; Shih et al., 2000). HNF4α is a major regulator in the hepatocyte and may control hundreds of genes coding for various metabolic processes (Odom et al., 2004; Kel et al., 2008). Hence, dysfunction of this transcription factor may be a predisposing risk factor for the development of NASH (i.e., by limiting apolipoprotein-mediated lipid clearance).

2. Lipotoxicity as a Mechanism of Steatohepatitis. *Lipotoxicity* refers to a cellular dysfunction due to intracellular overload of lipids. It was first proposed by Lee et al. (1994), who discovered that free fatty acids caused toxicity in pancreatic β-cells, impairing their capability to sufficiently secrete insulin. These and related findings finally provided the missing link between peripheral insulin resistance and the development of insulin-dependent diabetes type 2. In particular, it was hypothesized that increased exposure to FA during insulin resistance results in an impaired insulin secretion of pancreatic β-cells, which were found particularly sensitive to the fatty acid-induced disruption of cell metabolism and initiation of cell death (Delarue and Magnan, 2007). Besides detrimental effects in pancreatic β-cells, the concept of lipotoxicity has been translated to various tissues, including skeletal muscle, vascular endothelium, myocardium, and liver (Weinberg, 2006; Chinen et al., 2007). Lipotoxic effects of free fatty acids have been

suggested to be a key factor in development and also progression of hepatic steatosis (de Almeida et al., 2002; Malhi et al., 2006). In general, the term *lipotoxic effects* summarizes a potpourri of alterations in cellular metabolism observed in vitro upon addition of free fatty acids to cell cultures and includes 1) activation of stress-related signaling of JNK, 2) elevated expression of proinflammatory cytokines, 3) inhibition of mitochondrial β-oxidation, 4) elevated production of ROS, as well as enhanced generation of 5) toxic lipid intermediates and 6) lipid derivatives involved in altered cell signaling (Shimabukuro et al., 1998; Reddy, 2001; Borradaile et al., 2006b; Di Paola and Lorusso, 2006; Malhi et al., 2006). Saturated FA (i.e., palmitate) are the primary and most potent elicitors of lipotoxic effects (Eitel et al., 2002; Maedler et al., 2003; Weigert et al., 2004).

In vivo, detrimental effects of elevated free fatty acids particularly contribute to an inflammatory reaction observed in adipose tissue, obesity, and NASH, characterized by elevated plasma levels of TNF-α (Kern et al., 1995; Crespo et al., 2001; Valenti et al., 2002; Cai et al., 2005). Generation of oxidative stress is an important factor in lipotoxicity by virtue of its contribution to cellular stress signaling and interference with mitochondrial functions (Srivastava and Chan, 2007). In a recent report, palmitate was demonstrated to limit GSH synthesis by inhibition of cysteine transporter xCT and subsequently limited substrate supply (Srivastava and Chan, 2008). Thus, in addition to increasing intracellular levels of ROS, saturated fatty acids may also negatively affect the intracellular redox state by limiting the GSH-mediated oxidative defense. In particular, the ER (Borradaile et al., 2006a,b; Karaskov et al., 2006; Wei et al., 2006), mitochondrion (Maestre et al., 2003; Boudina et al., 2007; Srivastava and Chan, 2007; Koshkin et al., 2008), and lysosomes (Feldstein et al., 2004, 2006) were suggested as putative sites for lipotoxic stress induced by saturated free fatty acids. Finally, fatty acids were shown to induce apoptosis, an endpoint of lipotoxicity that is termed *lipoapoptosis* (Unger and Orci, 2002).

It was demonstrated that saturated fatty acids stimulated the expression of the gene coding for a proapoptotic member of the Bcl-2 family, namely Bim (Bcl-2-interacting mediator of cell death), which initiates apoptosis by inducing release of cytochrome *c* followed by activation of caspases 3 and 7 (Willis and Adams, 2005; Malhi et al., 2006). The effects of palmitic and stearic acid were observed to be dose-dependent and involved activation of JNK and proapoptotic protein Bax. Knockdown of Bim mRNA by the use of small interfering RNA consistently interrupted fatty acid-induced apoptosis, whereas JNK deficiency conferred resistance against FFA-induced apoptosis (Malhi et al., 2006). Saturated FFA-mediated induction of Bim was demonstrated to result from transcriptional activation by Foxo3a, which upon dephosphorylation via protein phosphatase 2 translocated into the nucleus and bound to the Bim promoter, as confirmed in a chromatin immunoprecipitation assay (Barreyro et al., 2007).

Ceramide signaling and particularly de novo synthesis of ceramide, which belongs to the class of sphingolipids, is thought to be of key importance in lipoapoptosis, which is induced by up-regulation of serine palmitoyltransferase (SPT) (Unger and Orci, 2002). It is noteworthy that dietary fatty acids and drugs were found to control SPT activity via substrate supply and may thereby enhance ceramide-mediated lipotoxicity (Merrill, 2002). Furthermore, cytokines, death receptor ligands, or xenobiotics may trigger ceramide formation by hydrolysis of sphingomyelin at various subcellular locations (van Meer and Holthuis, 2000). Such TNF-α, CD40 ligands, and other cytokines increase intracellular ceramide availability by activation of neutral and acidic sphingomyelinases (SPMase), the latter being activated by 1,2-diacylglycerol (Geilen et al., 1997; Kolesnick and Krönke, 1998).

A positive correlation between triacylglycerol accumulation and ceramide levels in livers of *ob*/*ob* mice was recently shown in a concerted approach to investigate the lipidome (Yetukuri et al., 2007). The exact mechanisms underlying lipid-induced apoptotic cell death by ceramide signaling has not been fully elucidated (Unger and Orci, 2002). Ceramide was found to interact with intrinsic (e.g., mitochondrially targeted) as well as extrinsic and death receptor-mediated apoptotic pathways (Ruvolo, 2003). Evidence has been put forward that ceramide induced apoptosis by permeabilizing the mitochondrial outer membrane to apoptosis-inducing proteins (Siskind et al., 2002, 2006, 2008; Stiban et al., 2008). In addition, inhibition of the mitochondrial respiratory chain complex 3 (Gudz et al., 1997) and of PI/Akt kinase activity have been discussed as probable mechanisms for an involvement of ceramide in induction of apoptosis (Zhou et al., 1998) (Fig. 5).

Enrichment of ceramide in mitochondrial membranes may also play an important role for ceramide-induced apoptosis and may occur directly before apoptosis followed by the formation of channels in mitochondrial outer membranes (Siskind et al., 2002, 2006). This enrichment of ceramide may be facilitated via mitochondria-associated membranes that are formed in the ER and, in contrast to ultrapurified mitochondria, contained dihydroceramide desaturase—an enzyme that generates ceramide from dihydroceramide. Incubation experiments with cellular subfractions and radiolabeled ceramide supported this hypothesis and documented an enrichment of ceramide in mitochondria, resulting in release of cytochrome *c*. Another interesting finding is that mere proximity between ER and mitochondrial subfractions is apparently sufficient to transfer ceramide and to induce mitochondrial permeabilization (Stiban et al., 2008). All together, this suggests that altered intracellular transport of ceramide may represent an alter-

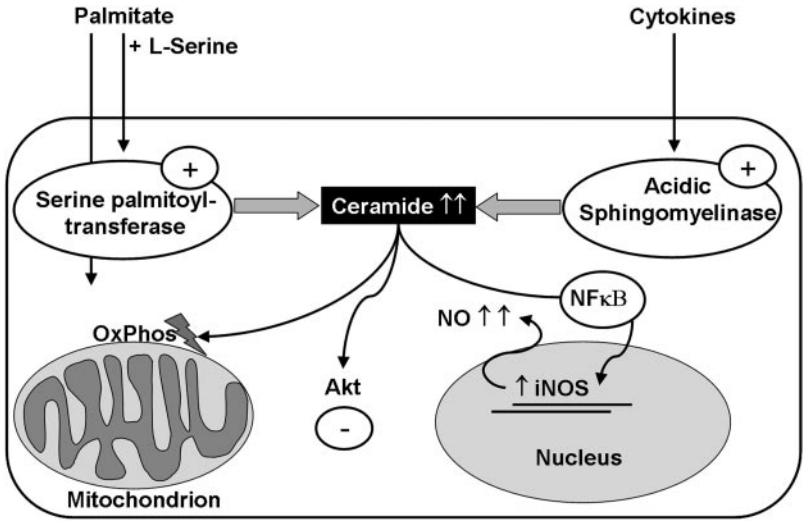

FIG. 5. Fatty acids induce de novo synthesis of ceramide via serine palmitoyltransferase, whereas cytokines cause ceramide level elevations by activation of acidic sphingomyelinase. Proposed roles of ceramide in lipoapoptosis are induction of *iNOS* gene expression via NF-κB associated with increased production of NO, inhibition of Akt activity, and direct inhibition of mitochondrial respiratory chain complex 3 (Unger and Orci, 2002).

native route to de novo ceramide synthesis for causing ceramide-induced lipoapoptosis.

A causal relationship, however, between ceramide and lipoapoptosis in case of hepatic steatosis remains to be established. In fact, experimental evidence suggested that lipotoxic effects of saturated fatty acids in hepatocytes occur in a ceramide-independent fashion in vitro (Barreyro et al., 2007). As such, FoxO3a-mediated induction of proapoptotic protein Bim in response to treatment with palmitic acid could not be interrupted by the ceramide synthase inhibitor fumonisin B1 (Barreyro et al., 2007). Consequently, it was concluded that FFA-induced lipoapoptosis and the associated stimulation of FoxO3a-dependent Bim expression occurred independently of ceramide. However, ceramide is a potent activator of protein phosphatase 2A (Ruvolo et al., 1999) and it was demonstrated that inhibition of ceramide synthase by fumonisin B1 in vivo results in an activation of other sphingolipid-metabolizing systems (e.g., SPMase and SPT), which are likely to contribute to an outbalance of the sphingolipid metabolism (He et al., 2006). It is therefore possible that increased activity of SPMase could compensate for reduced cellular ceramide levels by production of ceramide via sphingomyelin hydrolysis. Because the aforementioned experiments did not demonstrate that ceramide levels decreased in response to fumonisin B1 treatment, the involvement of ceramide in Fox03-dependent Bim expression and hence in hepatic lipoapoptosis cannot be excluded. Furthermore, lysophosphatidylcholine has been suggested as an alternative effector of lipoapoptosis. Inhibition of the Ca^{2+}-independent phospholipase A_2, the enzyme that generates lysophosphatidylcholine (LPC) by small interfering RNA reversed palmitate-induced elevations of intracellular LPC, was found to sufficiently inhibit palmitate-induced lipoapoptosis of hepatocytes (Han et al., 2008).

Lipotoxicity is therefore a significant mechanism in the development of steatosis and its progression to steatohepatitis. Lipoapoptotic cell death provides an explanation for elevated apoptosis rates in NASH livers and links hepatic lipid accumulation to inflammation. Immunocompetent cells are attracted and stimulated by recognition of factors that are released by apoptotic but also necrotic hepatocytes in NAFLD, such as MCP-1 and or high mobility group box protein-1, an alarmin that is released during ischemia or necrosis but not apoptosis (Tsung et al., 2007; Klune et al., 2008). Clinical data support increased apoptotic hepatocytes in patients diagnosed with NAFLD (Feldstein et al., 2004; Ribeiro et al., 2004; Ramalho et al., 2006). Furthermore, treatment with an oral caspase inhibitor reduced aminotransferase levels in patients diagnosed with liver diseases associated with increased apoptosis rates (i.e., hepatitis C) as well as in the small number of patients with NASH included in this study (Pockros et al., 2007). Cytokeratin 18 in biopsies or serum levels of its soluble form have been proposed as biomarker to determine severity of the disease or even differentiate between steatosis and steatohepatitis (Wieckowska et al., 2006; Yilmaz et al., 2007).

3. Nonalcoholic Hepatic Steatosis and Steatohepatitis—A Lipid Storage Disease? Besides an increased availability of circulating NEFA, which has been proposed to be a major determinant in the development of hepatic steatosis and nonalcoholic fatty liver disease (Lavoie and Gauthier, 2006), there is evidence that fatty acid uptake in the liver is enhanced by other molecular alterations in the regulation of fatty acid uptake (Bradbury and Berk, 2004; Berk et al., 2005; Chabowski et al., 2007).

Fatty acids are incorporated by a transmembranous flip-flop mechanism, and by mechanisms involving members of the fatty acid binding protein family (e.g.,

fatty acid binding protein 2 and 5 in the liver), mainly involved in uptake of long-chain fatty acids (C12-C20) (Stahl et al., 2001; Pohl et al., 2002, 2004; Ehehalt et al., 2006). Over expression of hepatic fatty acid translocase FAT/CD36, for instance, was associated with hepatic steatosis and has been implicated in steatosis augmented by LXR (Degrace et al., 2006).

In hepatocytes, uptake as well as lipolysis of lipids was mediated by caveolae (Cohen et al., 2004; Pol et al., 2004). Thus, attenuation of lipid raft trafficking provides another possible mechanism by which hepatic lipid uptake may be altered or lipid release may be impaired. Besides changes in lipid uptake that may predispose to hepatic steatosis, there is clinical evidence for altered lipid clearance via lipoproteins. Livers of patients with NASH displayed aberrations in VLDL metabolism and reduced excretion of lipids in livers of patients with steatosis and NASH (Charlton et al., 2002; Mensenkamp et al., 2004). Thus it was reported that in contrast to healthy and lean subjexts, patients with NAFLD displayed markedly altered VLDL secretion rates, as determined by turnover of apolipoprotein B (Charlton et al., 2002). It is noteworthy that ApoB-containing VLDL particles are assembled in a process that includes at least two stages. During the initiating step, ApoB is folded and stabilized, involving interactions with membrane lipids of the endoplasmic reticulum to eventually form VLDL precursors (Rustaeus et al., 1998). These precursors are loaded with different lipid classes during the maturation process (Charlton et al., 2002). VLDL synthesis and secretion is foremost dependent on substrate availability, which is determined by levels of SREBP-1c-dependent key lipogenic enzymes (Horton, 2002). Furthermore, activity of MTP determines VLDL synthesis (Shoulders and Shelness, 2005). In the absence of this enzyme, formation of VLDL precursors is inhibited, and VLDL secretion is impeded (Horton et al., 2003a).

In patients with NAFLD, the absolute ApoB secretion rate was significantly reduced, which is likely to contribute to an increase in hepatocellular lipid content (Charlton et al., 2002). In addition, a failure in insulin-mediated suppression of VLDL secretion as well as overproduction of large VLDL particles was observed in fatty livers of patients with T2DM (Adiels et al., 2006; Adiels et al., 2007). Taken together, these findings are suggestive for alterations of VLDL metabolism in hepatic steatosis and NAFLD but are not conclusive. Inhibitory effects of insulin on hepatic VLDL secretion so far have been demonstrated in patients and in animal models (Patsch et al., 1986; Brown and Gibbons, 2001). Therefore, failure to adapt VLDL secretion in patients with high liver fat content is likely to be the result of insulin resistance due to hepatic lipid accumulation. Reduced ApoB secretion, however, was also noted in in vitro experiments and animal models in response to challenge with high concentrations of fatty acids (e.g., oleic acid) (Sparks et al., 1997; Zhang et al., 2004). This was attributed to an ER stress-related increase in apolipoprotein B100 degradation through both proteasomal and nonproteasomal pathways (Ota et al., 2008). Thus induction of lipid-induced ER-associated degradation of ApoB100 may provide a further molecular explanation for reduced VLDL secretion in hepatic steatosis. In addition, direct oxidative damage to ApoB100 was suggested to promote its degradation via enzymatic or nonenzymatic pathways (Grune et al., 1997; Pan et al., 2004).

Indeed, an initially increased hepatocytic ApoB100 synthesis was observed upon challenge with oleic acid and triglyceride-derived fatty acids but seemed to exhaust after approximately 9 h under various experimental conditions (Ota et al., 2007). Reduction of ApoB100 secretion coincided with increases in ER stress markers, suggesting that although an initial adaptation in lipid-burdened hepatocytes is expected, ER stress could be a mechanism responsible for failure to appropriately enhance lipid clearance. This notion is further corroborated through experimental findings that demonstrated stimulation of VLDL secretion in response to increased fatty acid delivery to hepatocytes and triacylglycerol secretion both in vitro and in vivo (Fisher and Ginsberg, 2002; Zhang et al., 2004). 5-Lipoxygenase (5-LO) has emerged as a possible steatogenic factor that has been linked to impaired hepatic MTP activity and secretion of VLDL-TAG and ApoB. In particular, hepatic 5-LO-derived product levels were elevated, but inhibition of 5-LO activity restored hepatic MTP activity in parallel with a stimulation of hepatic VLDL-TAG and ApoB secretion in livers of *ob/ob* mice (López-Parra et al., 2008).

Besides VLDL assembly and secretion, other cellular lipid transporters, such as those of the ABC transporter family (e.g., ABCA1) may be involved in hepatic lipid accumulation. ABCA1 mediates the cellular phospholipid and cholesterol release and together with ApoAI is involved in HDL formation. Patients with mutations in the *ABCA1* gene suffer from familial HDL deficiency syndrome such as classical Tangier disease, resulting in low HDL plasma levels and defective reverse cholesterol transport to the liver (Fredenrich and Bayer, 2003; Kolovou et al., 2006). Several other members of the ABC transporter family are involved in maintenance of the cellular lipid homeostasis, such as ABCB1 (also known as MDR1), which regulates the phosphocholine export from hepatocytes into bile canaliculi, thereby controlling the phosphocholine (PC)/phosphoethanolamine (PE) ratio. A decreased PC/PE ratio is associated with liver damage, and normalization of PC/PE ratio has been reported to attenuate liver damage in a transgenic mouse strain with dietary steatohepatitis (Li et al., 2006).

Furthermore, activity of hepatic lipases or MTP may be limiting factors for the proper clearance of hepatic lipids (Sugimoto et al., 2002; Boucher et al., 2007). Inhibition of MTP activity was suggested to contribute,

at least in part, to lipid accumulation in alcoholic steatosis (Sugimoto et al., 2002), hepatitis C-related steatosis (Perlemuter et al., 2002), and drug-induced steatosis induced by amineptine, amiodarone, pirprofen, tetracycline, and tianeptine (Kulinski et al., 2002; Lettéron et al., 2003). MTP activity was significantly impaired by amiodarone, a drug known to cause steatohepatitis, at a concentration of 1 mM in vitro and reduced VLDL secretion in vivo (Lettéron et al., 2003). Inhibition of MTP as therapeutic concept in homozygous familial hypercholesterolemia reduced effectively elevated plasma low-density lipoprotein cholesterol and production of ApoB in patients but caused hepatic lipid accumulation and elevated liver transferase levels, again emphasizing MTP activity to be an important determinant for the development of hepatic steatosis (Cuchel et al., 2007).

In addition, genetic risk factors may sensitize for alterations in lipid secretory pathways, and functional polymorphisms of MTP have been suggested as risk factors for the development of steatosis and NASH (Björkegren et al., 2002; Gambino et al., 2007). Future research will be required to define the relevance of lipid export deficiencies as causative mechanisms for lipid accumulation in steatosis.

4. Organelle Toxicity in Nonalcoholic Hepatic Steatosis and Steatohepatitis. The two-hit hypothesis in NASH pathogenesis, originally proposed by Day and James (1998), considers extensive lipid accumulation to be the first hit, thereby activating different signaling pathways to cause perturbation of metabolic pathways and to increase vulnerability toward cellular injury, as will be discussed later on. The second hit is believed to result from an increase in oxidative stress, for instance due to uncoupling of the respiratory chain (Berson et al., 1998). In fact, oxidative stress activates a variety of proinflammatory stimuli, such as secretion of proinflammatory cytokines (e.g., TNF-α), chemokines, proliferation of stellate cells (also known as oval cells or Ito cells), and expression of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), E-selectin, or P-selectin, which promote adhesion and infiltration of polymorphonuclear cells (Lee et al., 1995; Jaeschke, 2000; Robertson et al., 2001; Capanni et al., 2006). Note that serum ICAM-1 levels were significantly elevated in patients with NASH and patients diagnosed with NAFLD compared with healthy subjects (Ito et al., 2007). The importance of oxidative stress in vivo is further supported by findings in animal models and clinical studies suggesting that treatment with antioxidants, e.g., vitamin E, may alleviate steatosis and the extent of liver injury (Robertson et al., 2001; Dufour et al., 2006; Portincasa et al., 2006; Yakaryilmaz et al., 2007).

Mitochondria are a significant source of ROS production and an obvious target of lipotoxicity and xenobiotic- or drug-induced toxicity (Pessayre et al., 2002; Zhang et al., 2007; Serviddio et al., 2008b). Excessive generation of ROS itself may be considered as a direct cause for mitochondrial dysfunction, because ROS interfere with the mitochondrial respiratory chain and integrity of mitochondrial DNA (Garcia-Ruiz and Fernandez-Checa, 2006). Particularly in primary NAFLD, mitochondrial dysfunction was proposed to be an indicator of advanced steatosis and progression to steatohepatitis (Garcia-Ruiz and Fernandez-Checa, 2006). Mitochondria in livers of patients with NASH are frequently marked by ultrastructural lesions, including crystalline inclusions, enhanced formation of oversized megamitochondria, and often display oxidative degeneration of mitochondrial DNA and oxidatively modified proteins (Pessayre et al., 2001; Sanyal et al., 2001; Pérez-Carreras et al., 2003). Clinical evidence confirms that activities of mitochondrial respiratory chain (MRC) complexes are decreased in livers of patients with NASH compared with healthy subjects (Pérez-Carreras et al., 2003). This mitochondrial dysfunction was positively correlated with serum TNF-α, insulin resistance, and BMI values (Pérez-Carreras et al., 2003). Likewise, reduced MRC activity was reported from studies with *ob/ob* mice (García-Ruiz et al., 2006). Mitochondrial dysfunction may be causally related to the development of steatosis, because compromised mitochondrial fatty acid oxidation is sufficient to induce hepatic steatosis. This is observed in patients with genetic defects in mitochondrial acyl-CoA dehydrogenases and in transgenic animal models with deficiencies in enzymes of mitochondrial fatty acid oxidation, both resulting in severe hepatic steatosis and steatohepatitis as a result of impaired fatty acid oxidation (Tolwani et al., 2005; Grosse et al., 2006; Zhang et al., 2007).

Inhibition of the mitochondrial fatty acid transporter CPT-1 (a rate-limiting step for fatty acid oxidation operating by transportation of long-chain fatty acids across the outer mitochondrial membrane into the matrix) by etomoxir is associated with development of steatosis and steatohepatitis (Koteish and Diehl, 2001). Likewise, inhibition of CPT-1 by increased levels of its endogenous inhibitor malonyl-CoA was suggested as a possible mechanism for hepatic lipid accumulation in patients with obesity as well as in patients with hepatic steatosis induced by the estrogen antagonist tamoxifen (Lelliott et al., 2005; Bandyopadhyay et al., 2006).

Furthermore, impairment of mitochondrial oxidative phosphorylation (OXPHOS) and electron transport may contribute to the development of hepatic steatosis by subsequently inhibiting β-oxidation (Grieco et al., 2005). It is noteworthy that the steatotic antianginal drugs perhexiline and amiodarone are cationic amphiphilic drugs that exert dual effects in mitochondrial respiration: transient uncoupling and subsequent inhibition of the electron transfer complexes I and II (Fromenty et al., 1990a). Both have been found to inhibit mitochondrial acyl-CoA dehydrogenase, CPT-1, and CPT-2 as well (Fromenty et al., 1990b; Kennedy et al., 1996).

Besides oxidative stress, several other causes may account for a failure of mitochondrial function, including 1) exposure to elevated TNF-α levels (Lee et al., 1999), 2) induction of ER stress and subsequent uncoupling protein (UCP)-2 expression (Nakatani et al., 2002; Ota et al., 2007), 3) ceramide-related impairment of OXPHOS (Hickson-Bick et al., 2000; Sparagna et al., 2000), 4) toxic fatty acid intermediates (Hashimoto et al., 1999; Echtay et al., 2003), 5) depletion of mitochondrial GSH (Garcia-Ruiz and Fernandez-Checa, 2006), and 6) altered mitochondrial membrane compositions (Colell et al., 2003; Marí et al., 2006).

TNF-α is a common factor in both primary and secondary NAFLD and is positively correlated with ongoing liver damage and inflammation. Elevated TNF-α levels are related to increased production in adipose macrophages (Maeda et al., 2002; Masaki et al., 2004), and Kupffer cells (Rose et al., 1997, 2001; Cai et al., 2005; Tomita et al., 2006). Treatment with TNF-α caused functional and morphologic alterations in mitochondria in vitro after incubation for 8 h (Sánchez-Alcázar et al., 2000). In contrast, treatment with the peroxynitrite scavenger uric acid and anti-TNF-α antibodies improved mitochondrial respiration, inflammation, and alleviated hepatic steatosis in mouse models of NASH (Li et al., 2003; García-Ruiz et al., 2006). It was therefore suggested that TNF-α might contribute to elevated mitochondrial peroxynitrite levels in NAFLD by inducing expression of iNOS in an NF-κB-dependent fashion (García-Ruiz et al., 2006; Yang and Rizzo, 2007). Nitric oxide as well as peroxynitrite may interfere with MRC components, thereby impairing mitochondrial OXPHOS (Radi et al., 2002b; Radi et al., 2002a; Murray et al., 2003). The role of TNF-α in NAFLD-associated liver injury is unclear. Evidence from in vitro studies indicated that TNF-α alone is not responsible for liver cell damage (Schrem et al., 2006), but may weaken liver cells, as detailed above, and attracts immune competent cells. The latter, namely CD8+T-cells and natural killer cells, are thought to cause hepatocytic damage by direct cytotoxicity and by inducing collateral damage through interaction with other leukocytes (Murray and Crispe, 2004).

Although NF-κB is initially thought to protect hepatocytes from oxidative stress and TNF-α induced cell death by induction of antiapoptotic proteins (Liu et al., 2002; Geisler et al., 2007), prolonged activation of the downstream signaling molecule JNK was found to promote inflammation and apoptosis (Chen et al., 1996). Phosphorylation of JNK by MAPK kinase 1 and apoptosis signaling kinase is initiated by the complex of TNF receptor adaptor proteins (Schwabe and Brenner, 2006). In particular, TNF-α-dependent generation of ROS may result in prolonged activation of JNK by inactivating MAPK phosphatases that otherwise would dephosphorylate and inactivate JNK (Kamata et al., 2005). So far, the distinct roles of the two JNK isoforms expressed in the liver (JNK1 and JNK2) in TNF-α induced cell death are not completely understood; however, studies in JNK1 and -2 knockout mice indicated that JNK2 may be important for activation of caspase 8 and mitochondrial pathways of apoptosis in response to TNF-α (Sabapathy et al., 2004; Wang et al., 2006b). In contrast, inhibition of JNK1 was shown to protect from TNF-α-induced and fatty acid-induced cell death (Schwabe and Brenner, 2006; Pagliassotti et al., 2007).

Feldstein et al. (2004) put forward a hypothesis to explain the involvement of free fatty acids in TNF-α formation in the liver. According to this hypothesis, the lysosome is a primary target of lipotoxic effects. The authors had reported that a high-fat diet caused intrahepatic lipid accumulation and translocation of the proapoptotic factor bax to the lysosome to subsequently induce release of lysosomal cysteine protease cathepsin B, which was responsible for degradation of IKK-β and caused activation of NF-κB. Upon translocation into the nucleus, NF-κB-dependent transcription of TNF-α was induced (Fig. 6).

Indeed, studies with MCD diet-fed mice showed reduced hepatocyte apoptosis and liver damage after cathepsin B inhibitor treatment, and cathepsin B knockout mice consistently displayed attenuated liver damage compared with wild-type mice, when they were exposed to cold ischemia-warm reperfusion (Baskin-Bey et al., 2005). The observation that lipoapoptosis in cathepsin B knockout mice was inducible to the same extent as in wild-type mice, however, indicated that free fatty acid-induced apoptosis may not necessarily be associated with Bid cleavage and release of cathepsin B (Malhi et al., 2006).

5. Microsomal Monooxygenases in Nonalcoholic Fatty Liver Diseases. Microsomal monooxygenases have been implicated as another putative source for excessive ROS formation in the pathogenesis of NAFLD. Induction of CYP2E1, for instance, and subsequent production of ROS were reported for primary NAFLD as well as

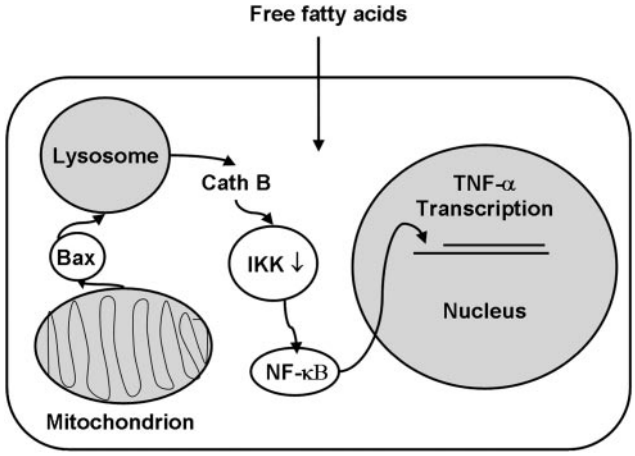

FIG. 6. A simplified scheme of the mechanism proposed by Feldstein et al. (2004), by which lipids lead to activation of TNF-α. Cath B, cathepsin B.

drug- and alcohol-induced liver diseases (Niemelä et al., 2000; Lieber, 2004; Bai and Cederbaum, 2006a,b; Lu and Cederbaum, 2006). CYP2E1 is induced by several substrates, including fatty acids, ketones, ethanol, and xenobiotics and is frequently induced in steatosis. As a result of futile cycling of substrates in monooxygenases, particularly CYP2E1, cytochrome activity is associated with release of free radicals, which cause lipid peroxidation (Lieber, 1997). Observations that CYP2E1 knock-out mice also display massively elevated lipid peroxidation during hepatic steatosis with concomitant up-regulation of CYP4 isoforms A10 and A14, however, led to the conclusion that induction of CYP2E1 is not necessarily required for lipid oxidation in NASH (Leclercq et al., 2000). The intracellular origin, which is the initial source of ROS generation during steatosis, remains uncertain.

Oxidative stress alone may or may not be sufficient to cause liver injury in steatosis. It is noteworthy that although oxidative DNA damage occurs already at early stages of steatohepatitis, up-regulation of adaptive antioxidant mechanisms preserved the viability of hepatocytes in vitro (Cortez-Pinto et al., 1999; Rashid et al., 1999; Diehl, 2005). It was demonstrated that induction of the mitochondrial UCP-2, which is a possible cellular mechanism to adapt to ROS and to prevent apoptosis by uncoupling the oxidative phosphorylation, caused enhanced susceptibility of hepatocytes to other stressors, such as hypoxia, TNF-α, or carbon tetrachloride-induced toxicity (Cortez-Pinto et al., 1998; Yang et al., 2000; Garcia-Ruiz and Fernandez-Checa, 2006; Donthamsetty et al., 2007; Park et al., 2007). Activation of UCP-2 by FA hydroperoxides, which are products of lipid peroxidation, is thought to be part of a negative feedback mechanism to reduce intracellular ROS (Jezek et al., 2004). Note that in contrast to accidental interruption of the respiratory chain by steatotic drugs, coordinated and slight uncoupling of the oxidative phosphorylation by UCP-2 allows backflow of H^+ into the mitochondrial matrix and substantially reduces formation of oxidative species. This is achieved by a slightly enhanced respiration and a subsequently reduced oxygen tension and shortened lifetime of ubiquinone anion radical (Jezek et al., 2004). Adaptive mechanisms to oxidative stress were also reported for livers of patients with NASH (Perlemuter et al., 2005; Park et al., 2007). Mitochondria obtained from patients with NASH were confirmed to display UCP-2 expression, elevated ROS level and increased levels of lipid peroxidation products (Serviddio et al., 2008a). An increased sensitivity of NASH livers to stressors was demonstrated in animal models of NASH, which displayed significantly increased mitochondrial ROS production and impaired ATPase activity in response to ischemia-reperfusion injury (Serviddio et al., 2008a).

Thus, the role of oxidative stress in steatosis and steatohepatitis has been suggested to lie in an increased vulnerability of cells to other stressors, such as toxic effects of drugs in drug-induced liver disease or fatty acids in obesity and NASH (Diehl, 2005). This may explain the findings that steatotic livers of *ob/ob* mice failed to recover ATP levels after hypoxia compared with lean mice (Chavin et al., 1999). At this point, it should also be noted that hepatic microcirculation is impaired by severe steatosis and may thereby significantly increase liver injury in steatotic livers exposed to secondary insults (Sun et al., 2001; Hasegawa et al., 2007).

6. Endoplasmic Reticulum in Steatosis. Induction of ER stress has been reasoned as a potential mechanism promoting progression of hepatic steatosis, by worsening the cellular energy situation and fanning biosynthesis and uptake of cholesterol and triacylglycerols. This notion has been supported in the model of hepatic steatosis in patients with hyperhomocysteinemia. This condition arises from deficiencies in vitamins, other essential nutritional factors (B6, B12, folic acid, betaine) or genetic defects of cystathionine β-synthase or 5,10-methylene tetrahydrofolate reductase (MTHFR), resulting in accumulation of homocysteine in the body, which is frequently associated with development of hepatic steatosis and cardiovascular complications (Ji and Kaplowitz, 2004). Alterations in cholesterol and triglyceride metabolism were observed in homocysteine-induced ER in cultured human cells and murine models of hyperhomocysteinemia (Doerrler and Lehrman, 1999; Werstuck et al., 2001). In particular, homocysteine-induced endoplasmic reticulum (ER) stress was reported to activate both the unfolded protein response and the sterol regulatory element-binding proteins (SREBPs) in cultured human hepatocytes (Werstuck et al., 2001).

Recent findings revealed that the ER is a target to lipotoxic stress primarily mediated by the increased production of ROS (Borradaile et al., 2006a,b). Addition of palmitate to cell cultures altered the lipid composition of the ER membrane toward an increased saturation degree, which preceded apoptosis possibly induced through calcium flux from the ER to mitochondria and subsequent mitochondrial permeability transition (Demaurex and Distelhorst, 2003; Rao et al., 2004). Studies with hepatocyte cultures and in vivo experiments in mice demonstrated that oxidative stress and lipid oxidation-induced post-ER presecretory proteolysis to be responsible for the degradation of apolipoprotein B100 and reduced VLDL secretion (Pan et al., 2004). These findings suggest that induction of ER stress may significantly contribute to the pathogenesis of NAFLD.

7. Endocrine Mediators and Signaling Networks in Hepatic Steatosis. Adipocyte hormones, such as adiponectin, leptin, and resistin, that travel between adipocytes and liver have been found to play an important role in the regulation of hepatic lipid metabolism and to be key players in the pathogenesis of steatosis.

Adiponectin is an adipocyte hormone that is expressed exclusively in adipose tissue and is recognized by adi-

ponectin receptors 1 and 2) expressed in the liver (Yamauchi et al., 2001, 2003). The positive effect of adiponectin on lipid oxidation is mediated by activation of PPARα and phosphorylation of AMPK (Yamauchi et al., 2001, 2003). By inhibiting the rate-limiting enzyme in fatty acid biosynthesis acetyl-CoA carboxylase, AMPK lowers the concentration of malonyl-CoA and increases β-oxidation (Ruderman et al., 2003). Furthermore, adiponectin influences intracellular lipid processing by modulating the activity of AMPK, which regulates triglyceride and cholesterol synthesis via suppression of SRPBP-1 and ChREBP and apparently improves insulin signaling via IRS-1 (Foretz et al., 2005; Andreelli et al., 2006; Yoon et al., 2006; Li et al., 2007; Wang et al., 2007a). In addition, adiponectin was recognized as a modulator of inflammation by suppressing IKK-β activation induced by TNF-α (Wu et al., 2007). In particular, the uncleaved (high molecular weight form) of adiponectin was found to suppress cytokine-induced NF-$\kappa\beta$ activation in cardiac fibroblasts and endothelial cells, whereas the proteolytic cleavage product activated NF-$\kappa\beta$ and thereby promoted expression of pro-inflammatory and adhesion molecule (Hattori et al., 2007; Tomizawa et al., 2008). Clinical studies revealed that plasma adiponectin levels were lower in patients with nonalcoholic fatty liver disease compared with healthy subjects and were inversely correlated with grade of inflammation and extent of liver injury (Musso et al., 2005; Pagano et al., 2005; Aygun et al., 2006; Targher et al., 2006; Wong et al., 2006). Consequently, replenishing adiponectin levels by its recombinant substitute attenuated steatosis in overnutrition, obesity, and insulin resistance but also ameliorated chronic liver injury induced by ethanol and carbon tetrachloride, suggesting that adiponectin may play a central role in the development of hepatic steatosis and fibrosis (Kamada et al., 2003; Xu et al., 2003). The effects of adiponectin in the lipid metabolism of the liver are summarized in Fig. 7, below.

Although several studies connected adiponectin levels with the liver's ability to conduct lipid oxidation and limit lipogenesis, others reported a strong correlation between basal endogenous glucose production and plasma adiponectin levels in vivo (Ukkola and Santaniemi, 2002; Santaniemi et al., 2006). An involvement of adiponectin in the development of insulin resistance was further supported by the discovery of mutations of the adiponectin gene, which are clinically associated with hypoadiponectinemia and diabetes (Waki et al., 2003; Kadowaki and Yamauchi, 2005; Vaxillaire et al., 2006). In contrast, serum levels of the adipokine resistin are increased in patients with NASH and steatosis and correlated with hepatic fat content and hepatic insulin resistance (Bajaj et al., 2004; Pagano et al., 2006).

The strong insulin-sensitizing adipokine leptin was initially thought to play a major role in the development of insulin resistance and hepatic steatosis (Angulo et al., 2004; Ikejima et al., 2005). Investigations focusing on the relationship between leptin and steatosis revealed that serum leptin levels in patients with NASH were positively correlated with hepatic steatosis, fibrosis, and inflammation, as well as with serum lipids, glucose, insulin, c-peptide, and alanine aminotransferase (ALT) levels (Chitturi et al., 2002; Nobili et al., 2006). Studies in rodent models revealed that leptin prevented lipotoxicity by attenuating triglyceride accumulation in the liver, and activation of defective leptin signaling in *fa/fa* rats was found to massively reduce hepatic triglyceride contents (Chitturi et al., 2002; Leclercq et al., 2002; Unger, 2002). The experimental evidence presented, however, suggested that leptin plays a major role in insulin resistance by weakening lRS-1/IRS-2 signaling in the liver but is not necessary to induce steatosis (Benomar et al., 2005; Diehl, 2005). Instead, because of its potent effects on the immune system and promotion of inflammation, its role in the development of fibrosis

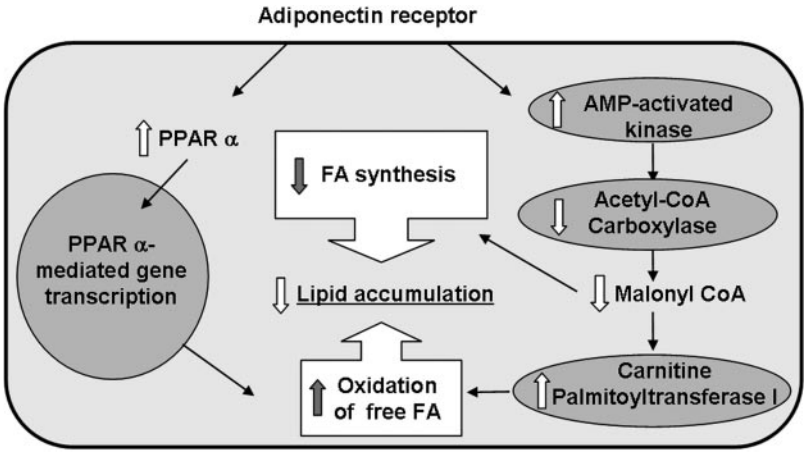

FIG. 7. Role of adiponectin in stimulating lipid oxidation and preventing lipid accumulation in the liver: through activation of signaling cascades and subsequently PPARα and AMPK-activated kinases, adiponectin triggers oxidation of fatty acids by both transcriptional and metabolic control (malonyl-CoA levels). [Adapted from You M, Considine RV, Leone TC, Kelly DP, and Crabb DW (2005) Role of adiponectin in the protective action of dietary saturated fat against alcoholic fatty liver in mice. *Hepatology* **42:**568–577. Copyright © 2006 John Wiley & Sons. Used with permission.]

and cirrhosis has been emphasized (Leclercq et al., 2002; Tsochatzis et al., 2006).

Aside from adipokines, recent reports suggested that patients with NAFLD may present a subclinical dysfunction of the HPA axis (Westerbacka et al., 2003; Targher et al., 2005; Cheung et al., 2007). Overall, the role of hormonal factors in the activation of lipogenic pathways and suppression of lipolysis on the molecular level of metabolically induced NASH has been repeatedly emphasized, but the mechanism by which hormones (i.e., angiotensin II, noradrenalin) (Marra et al., 2008) and adipokines, such as leptin, adiponectin, resistin, and the recently discovered lipocalin 2 (Yan et al., 2007), influence the development and progression of NASH remains far from clear.

E. Molecular Switches between Steatosis and Steatohepatitis

1. Cross-Talk of Hepatocytes with Stellate Cells, Fibroblasts, Endothelial, and Kupffer Cells in Progressive Liver Disease. There is growing evidence for extensive exchange of molecules among the different cell types of the liver, such as hepatocytes, endothelial cells, fibroblasts, cholangiocytes, stellate cells, and Kupffer cells. It is noteworthy that cross-talk between liver cells and other cell types, such as leukocytes, may be of great importance for activity and progression of NAFLD. Indeed, exchange and processing of proinflammatory signaling molecules seems to be essential for the progression of NAFLD and its associated architectural changes [i.e., fibrosis due to altered extracellular matrix production of transformed stellate cells to (myo)fibroblastic cells] (Diehl, 2002; Edwards et al., 2005; Bilzer et al., 2006; Yang et al., 2007).

From in vitro experiments it could be demonstrated that endothelial cells of the sinusoid required coculture with hepatocytes for the regulation of vascular cell adhesion molecule, ICAM, CD31, and E-selectin expression (Edwards et al., 2005). The cross-talk between these two cell types may be grossly changed during inflammation. In NAFLD, leukocytes adhere and migrate through the endothelial barrier to the space of Disse toward the liver cell parenchyma (Adams et al., 1994; Lalor et al., 2002) and are directed by cell adhesion molecules and chemotactic molecules. Nonetheless, it remains to be determined whether metabolically compromised and steatotic hepatocytes provide the initial signal for sinusoidal infiltration of immuno-component cells or whether other factors such as compression of the sinusoid as a result of hepatocyte enlargement foster leukocyte invasion into the fatty liver (Edwards et al., 2005; Bilzer et al., 2006; Thabut et al., 2007). In addition, endothelial dysfunction at the sinusoid may be a further confounder in NAFLD (i.e., due to increased systemic level of circulatory cytokines and LPS, increased levels of ROS, VEGF expression) (Spolarics, 1998). In this regard, VEGF and epidermal growth factor receptor may play important roles for the activation of the sinusoidal endothelium by affecting the nitric oxide (NO) equilibrium. Steatotic livers display a markedly reduced sinusoidal space (up to 50% of controls in animal models) as a result of compression by enlarged fat-loaded and hydropic (ballooning) hepatocytes as well as infiltration of macrophages (Farrell et al., 2008). This leads to a decreases in microvascular blood flow and causes sinusoidal deformation that can be visualized by in vivo microscopy (Farrell et al., 2008). This suggests that progression of hepatic lipid accumulation in steatosis alone may be sufficient to alter endothelial cell signaling toward leukocyte recruitment and activation.

Leukocyte activation is a basic program of pathogen defense and involves activation of NF-κB, JNK, and SOCS pathways via Toll-like receptors (Takeda and Akira, 2005). Stimulation of these programs in macrophages may contribute to collateral damage of adjoining parenchymal liver cells and is thought to be an important factor for disease progression (Rivera et al., 2007; Tu et al., 2008). It is of considerable importance that fatty acids [particularly saturated fatty acids (C14:0, C16:0, and C18:0)] were demonstrated to interfere with TLR 4 and may thereby contribute to pro-inflammatory stimulation of leukocytes or resident macrophages in hepatic steatosis (Shi et al., 2006; Shah, 2007).

Leukocytes have been reasoned to provide a significant origin for Kupffer cell (KC) activation in NAFLD by triggering KCs activation via interaction of CD11b/CD18 with ICAM-1 receptors at the KC surface and likely by release and subsequent activation of KC-expressed receptors for complement factors C3a and C5b (Bilzer et al., 2006). In particular, this recruitment and activation of KCs, which are the resident macrophages of the liver, has been recognized as a major determinant for the extent of liver injury (Andrés et al., 2003; He et al., 2005; Kresse et al., 2005; Malaguarnera et al., 2006a). A positive correlation between the activity of KC and steatohepatitis, as well as activation of stellate cells, elevated TNF-α levels and lipid peroxidation in patients with both NASH and simple steatosis could be established (Malaguarnera et al., 2006b). Thus Kupffer cell activation (i.e., the KC-produced enzyme chitotriosidase) was proposed as a diagnostic marker for monitoring steatosis and progression to steatohepatitis (Malaguarnera et al., 2006a,b; Kiki et al., 2007). Increased KC activation in hepatic steatosis, however, may also be induced by presence of phagocytosable particles (i.e., defect erythrocytes, oxidized low-density lipoprotein, VLDL), as well as release of ROS and antigens by apoptotic, hypoxic, or metabolically compromised hepatocytes (i.e., MCP-1, alarmins, complement factors C3 and C5), which are subsequently recognized by Kupffer cell receptors, such as TLRs (Nenseter et al., 1992; Korolenko et al., 2000; Ribeiro et al., 2004; Bilzer et al., 2006; Malaguarnera et al., 2006a; Shi et al., 2006; Otogawa et al., 2007).

Detrimental effects of Kupffer cells are attributed to production of ROS, which challenges the oxidative stress defense of cellular neighbors (Jaeschke and Farhood, 1991), as well as to release of pro-inflammatory cytokines, which by activation of hepatocellular receptors (i.e., IL-6 receptor, TNF-α receptors R1 and R2) induce specific hepatocellular programs, such as signal transducer and activator of transcription-3 and NF-κB signaling and/or apoptosis (Ding et al., 2003; Kresse et al., 2005; Bilzer et al., 2006; Yu et al., 2006). By recruitment of adaptor proteins such as TRADD, TRAF2, RIP, and FADD, TNF receptors activate NF-κB, JNK, and p38 and promote apoptosis by activation of caspases, mitochondrial depolarization, and subsequent release of cytochrome *c* (Schwabe and Brenner, 2006). Hepatic steatosis is a predisposing factor to TNF-α-induced cell death (Diehl, 2005). Nonetheless, KC function was found to be essential for hepatocellular proliferation and crucial in liver regeneration (Malik et al., 2002; Bilzer et al., 2006; Abshagen et al., 2007). In particular, hepatocyte growth factor as well as the cytokines IL-6 (Aldeguer et al., 2002) and TNF-α (Kirillova et al., 1999; Abshagen et al., 2007) provided by Kupffer cells and endothelial cells stimulate hepatocyte proliferation by triggering hepatocytic DNA replication via NF-κB (Seto et al., 1998; Armbrust et al., 2002; Bruun et al., 2002; Lalani et al., 2005; Bastard et al., 2006; Wang et al., 2006a; Simons et al., 2007). In addition, Kupffer cells seem to be involved in maintenance of adequate perfusion during liver cell proliferation—a function that could be related to Kupffer cell-derived cytokines or Kupffer cell-mediated effects on nitric oxide metabolism, which apparently plays an important role in hepatic microcirculation, particularly after liver injury (Rolfe et al., 1997; West et al., 1999; Holden et al., 2000; Meijer et al., 2000, Abshagen et al., 2008; Palmes et al., 2005; Schuett et al., 2007).

Cross-talk between different liver cell types seems to perpetuate hepatic steatosis. In particular, release of TNF-α and TGF-β1 triggers transformation of stellate cells from the vitamin A-storing phenotype to the myofibroblastic phenotype, resulting in increased production of collagen types I and III, which is necessary for fibrotic remodeling (Friedman, 1990; Meyer et al., 1990; Gressner and Weiskirchen, 2006; Tomita et al., 2006). Activation of stellate cells is also triggered by long-chain polyunsaturated fatty acids and ROS (Gressner and Weiskirchen, 2006; Urtasun and Nieto, 2007). These factors in particular could contribute to a constitutively lowered threshold for stellate cell activation in hepatic steatosis, such as that induced by obesity, fat-induced diets, or peripheral insulin resistance. In addition, recognition of hepatocytic DNA from apoptotic hepatocytes by stellate cells may provide the signal for the migration arrest that is required for differentiation of hepatic stellate cells (Watanabe et al., 2007). It is noteworthy that activation of stellate cells, in particular, has been evaluated as a critical factor of progression of fibrosis, because it has been associated with advanced disease in patients with NAFLD (Feldstein et al., 2005; Yoneda et al., 2007). This information besides its putative diagnostic value, suggests collagen synthesis as a therapeutic target in the treatment of advanced (fibrotic) NASH (Fiorucci et al., 2004, 2007; Kohjima et al., 2007). It is considerably important that composition of extracellular matrix is a critical determinant in the maintenance of the hepatocytic and sinusoidal phenotype (Hansen et al., 2006; Sellaro et al., 2007). In fact, qualitative and quantitative changes in ECM trigger stellate cell activation beyond other paracrine stimuli, thereby shifting the balance toward fibrosis (Sato et al., 2003; Zhou et al., 2006).

Intercellular communication in the liver may also be influenced by endocrine, paracrine, and even autocrine factors that may affect communication via gap junctions. Gap junctions are built from connexin proteins, which form intercellular channels, thereby connecting the cytoplasm of adjacent cells. Connexin 43, member of the connexin protein, facilitates exchange of small signaling molecules (i.e., Ca^{2+}, cAMP, inositol triphosphate) (Spragg and Kass, 2005) and was up-regulated in activated stellate cells (Fischer et al., 2005). Transcriptional regulation of connexin 43 is influenced by, for example, vitamin A, vitamin D3, LPS, thyroid hormone (T3), dexamethasone, plateled-derived growth factor, and endothelin-1, and interleukin 1-β may play a role in stellate cell activation (Fischer et al., 2005). Furthermore, a role for gap junction-mediated communication for the proper formation of fenestrae in endothelial cells was evidenced in transmission electron microscopic studies in an artificial liver organoid construct of immortalized sinusoidal endothelial and hepatic stellate cell lines (Saito et al., 2007). Thus, environmental factors, such as vitamins and hormones influence the intercellular communication of active stellate cells, suggesting a role in the coordination of fibrosis. In this regard, the renin-angiotensin-aldosterone system and, as recently discovered, the endogenous cannabinoid system may play important roles in the regulation of stellate cell activity (Jeong et al., 2008). The potent vasoconstrictor angiotensin II (ATII) was found to stimulate the tissue inhibitor of metalloproteinases-1 through PKC in activated stellate cells and was reported to induce expression of gap junction proteins (Dodge et al., 1998). Positive results in NASH-related hepatic fibrosis were reported upon treatment of MCD-fed rats with ATII blocker olmesartan, which significantly attenuated signs of liver injury, activation of hepatic stellate cells, oxidative stress, expression of collagen genes, as well as liver fibrosis (Hirose et al., 2007). Thus, antagonizing the ATII receptor may be a promising therapeutic strategy to reduce stellate cell activation and fibrotic remodeling in NASH (Yoshiji et al., 2007).

Finally, insulin resistance and effects of the adipokines leptin and adiponectin in the different liver cell types are currently under investigation. There is, how-

ever, evidence for a role of adiponectin expression, insulin resistance, and PPAR dysregulation in sinusoidal liver cells (Weglarz and Sandgren, 2004). Further evidence indicates a role for leptin signaling in Kupffer and hepatic stellate cells, thereby linking discrepancies in liver cell cross-talk to the metabolic background of NAFLD (Loffreda et al., 1998; Cao et al., 2007; Ikejima et al., 2007; Leclercq et al., 2007).

2. The Role of Arachidonic Acid, Cyclooxygenase II, and Arachidonic Acid Metabolites in Progressive Steatotic Liver Disease. Eicosanoids (e.g., prostanoids and leukotrienes), lipid mediators derived from long-chain polyunsaturated fatty acids [e.g., arachidonic acid, eicosapentaenoic acid, or docosahexaenoic acid (DHA)], have recently been identified and described to exert anti-inflammatory effects and to fulfill immunoregulatory functions (González-Périz et al., 2006; Schwab and Serhan, 2006). Because of their biological activity of resolving inflammatory processes and their neuroprotective abilities, lipid mediators derived from eicosapentaenoic acid and DHA have been termed resolvins and protectins (Chiang and Serhan, 2006). Lipoxins, which may originate from an interaction of endothelial cells and leukocytes, are derived from arachidonic acid and, together with the class of aspirin-triggered 15-epi-lipoxins, act as regulators of transendothelial-transepithelial polymorphonuclear leukocyte migration (Chiang and Serhan, 2006). In particular, secretion of lipoxins by one cell type and their modulation performed by another cell type, a process termed *transcellular biosynthesis*, may play an important role for cellular interactions and modulation of lipid signaling by switching the production of certain eicosanoid classes into another (Clària and Planagumà, 2005). In the liver, lipoxins are involved in dynamic interactions maintained between Kupffer cells and hepatocytes. It was demonstrated that biosynthesis of the aspirin-triggered lipoxins 15-epi-LXA_4 and LXA_4 involves the conversion of hepatocyte-derived 15-hydroxyeicosatetraenoic acid by the 5-LO of Kupffer cells (Clària and Planagumà, 2005). 15-epi-LXA_4 and LXA_4 have been found to modulate transcription factors (e.g., PPARα) and regulate cytokine production and thus the chemotactic properties of hepatic cells (Clària and Planagumà, 2005). Furthermore, lipoxins and ATLs inhibit inflammation, chemotaxis, selectin- and integrin-mediated adhesion, as well as transmigration across endothelial monolayers in human neutrophils. It is most likely that these lipid mediators take part in the coordination of Kupffer cell activities and hepatocytic signaling (Fierro et al., 2002; Titos et al., 2005; Chiang and Serhan, 2006; Schwab and Serhan, 2006). Based on these findings, involvement of these lipid mediators in the development and progression of NASH seems conceivable. In particular, altered availability of *n*-6 PUFAs, such as arachidonic acid, in progressive NAFLD may shift the balance of lipid signaling toward production of inflammatory eicosanoids, thereby possibly contributing to the development of steatohepatitis (Bagga et al., 2003; Videla et al., 2004; Elizondo et al., 2007).

It is noteworthy that evidence has been presented that Kupffer cell-derived factors (i.e., IL-6, PGE_2, TNF-α) may directly affect the lipid metabolism in hepatocytes in vitro and in vivo and may thereby contribute to intrahepatic lipid accumulation (Grunfeld et al., 1990, 1991; Adi et al., 1992; Enomoto et al., 2000, Brass and Vetter, 1994; Neyrinck et al., 2004). In a model of ethanol-induced steatosis, increased hepatic triglyceride contents correlated significantly with increased PGE_2 levels derived from Kupffer cells (Enomoto et al., 2000). Likewise, incorporation of [2-^{14}C]pyruvate into fatty acids (per mg protein) increased for up to 56% in cultures of primary rat hepatocytes after treatment with IL-6 (Brass and Vetter, 1994). Because TNF-α and IL-6 were demonstrated to enhance prostaglandin production in macrophages, release of these cytokines may aggravate hepatic lipid accumulation in the course of steatohepatitis (Peters et al., 1990; Ruhl et al., 1992). Further sensitization of Kupffer cells by ethanol, endotoxemia or ROS are likely to enhance PGE_2-mediated effects on hepatic lipogenesis (Enomoto et al., 1999; Ahmad et al., 2002; Dieter et al., 2002; Yamashina et al., 2005).

A role for cyclooxygenase 2 (COX-2)-derived PGE_2 in the progression of NAFLD is further indicated by findings in hepatic stellate cells, in which COX-2-derived PGE_2 was demonstrated to inhibit basal and TGFβ-1-mediated induction of collagen synthesis α 1(I), suggesting that KC-derived PGE_2 may thereby influence hepatic fibrosis (Hui et al., 2004). Abrogation of PGE_2 signaling by inhibition of COX-2 and subsequently suppressed production of PGE_2 was therefore suggested as a therapeutic principle for the treatment of steatosis. Results from studies in mouse models of steatosis confirmed that COX-2 inhibition protected against the development of steatohepatitis (Yu et al., 2006), whether these effects were related to KC-derived PGE_2, however, is not conclusive and may relate to COX-2 inhibition in other tissues. In this context, it is of considerable importance that PGE_2 was found to elicit positive effects on hepatocytes and sinusoidal cells in models of reperfusion/ischemia injury of the liver, where hepatic microcirculation was improved and liver injury was ameliorated by stimulation of prostaglandin E2 receptor EP4 (Arai et al., 1999; Kuzumoto et al., 2005). Others reported that PGE_2 exerted antiapoptotic and proproliferative effects via activation of EP4 in hepatocytes in vitro and of EP3 in vitro as well as in vivo (Arai et al., 1999; Kataoka et al., 2005; Casado et al., 2007; Meisdalen et al., 2007). The critical role for fatty acid signaling molecules in hepatic inflammation and fibrosis was stressed recently in a model of carbon tetrachloride-induced liver injury in which selective inhibition of COX-2 and 5-LO pathways independently reduced necroinflammation and fibrosis, but also resulted in an increase of apoptotic nonparenchymal liver cells (Hor-

rillo et al., 2007). Taken collectively, the roles of COX-2 and 5-LO pathways, and PGE_2 in particular, in the pathogenesis of NAFLD should be delineated in more detail before considering these as therapeutic targets.

III. Therapeutic Interventions

A. Diets and Insulin-Sensitizing Therapies

1. Weight Loss to Ameliorate Nonalcoholic Hepatic Steatosis and Steatohepatitis. Diets with and without additional exercising programs to restore insulin sensitivity were reported to improve the pathology of NASH. Positive effects in patients with NASH were achieved with regard to an improvement of serum transaminase levels (Kugelmas et al., 2003; Zhu et al., 2003; Hickman et al., 2004) and histopathological scores (Andersen et al., 1991; Dixon et al., 2004; Huang et al., 2005). In patients with obesity, dietary interventions associated with weight loss were accompanied by significant reductions of hepatic lipid contents, suggesting positive effects in the prevention of NAFLD (Petersen et al., 2005; Tamura et al., 2005; Westerbacka et al., 2005). Similar positive results were obtained with studies in which weight loss was induced by bariatric surgery (Clark et al., 2005; Mattar et al., 2005; Dixon et al., 2006; Dixon, 2007; Liu et al., 2007b). This increased insulin sensitivity also markedly improved the major components of the metabolic syndrome: glucose tolerance, hypertension, hypertriglyceridemia, and hypercholesterolemia (De Ridder et al., 2007). In a retrospective follow-up study, 312 patients with obesity and type 2 diabetes achieved most of their weight loss after the first year of bariatric surgery, which was paralleled by improvements of hyperglycemia and hypercholesterolemia (Scopinaro et al., 2005). Although the majority of patients achieved a major weight loss after the first year of surgery, positive effects on hypertriglyceridemia and arterial hypertension further improved over time (Scopinaro et al., 2005). In addition, weight loss due to bariatric surgery was associated with improved fibrosis and cirrhosis of the liver in patients with progressive NAFLD (Kral et al., 2004). Improvement of NASH by weight loss may also be the result of a reduction in systemic cytokine levels (McMillan, 1989; Das, 1999; Fernández-Real and Ricart, 2003). There are, however, some concerns about the effects of rapid weight loss, which was reported to enhance portal inflammation and fibrosis (Andersen et al., 1991). Results from follow-up studies were not conclusive about the risk of disease progression after bariatric surgery (Kral et al., 2004; Jaskiewicz et al., 2006; De Ridder et al., 2007; Furuya et al., 2007).

Pharmacological interventions achieved similar metabolic effects and positively influenced NASH (Derosa et al., 2004). The lipase inhibitor orlistat, which prevents lipid absorption, was found to reduce hepatic steatosis and fibrosis (Hussein et al., 2007) and was reported to positively influence serum ALT levels as well as steatosis, even if no weight loss was achieved (Zelber-Sagi et al., 2006). Weight loss as a result of administration of norepinephrine and the serotonin reuptake inhibitor sibutramine, which suppresses appetite and stimulates thermogenesis, was reported to decrease levels of γ-glutamyl transferase (GGT) and serum transaminases (Sabuncu et al., 2003).

Note that information on surrogate markers of disease (i.e., serum transaminase and GGT levels) presented with clinical data in this section have to be interpreted carefully. With respect to the long-term prognosis of hepatic function, these markers may be misleading, which will be discussed in the following. However, because there is no reliable marker to predict progression of NAFLD, reduction in intrahepatic lipid contents, insulin resistance, and inflammatory markers, as well as long-term improvement of the metabolic syndrome, may be considered positive predictors of disease.

2. Insulin Sensitizers in the Therapy of Nonalcoholic Hepatic Steatosis and Steatohepatitis. Insulin resistance may be treated with agents such as the thiazolidinediones (also known as glitazones; e.g., troglitazone and pioglitazone), or with metformin, which improves insulin sensitivity by acting on both hepatic glucose metabolism and glucose uptake and metabolism in skeletal muscle (Reynaert et al., 2005).

Metformin is used to treat T2DM in overweight patients. Metformin reversed insulin resistance, improved hepatic steatosis, and decreased TNF-α levels (Solomon et al., 1997; Marchesini et al., 2001b; Duseja et al., 2007). Experimental evidence was provided that reduction of intrahepatic lipid contents under metformin treatment could be explained by reduced activity of acetyl-CoA carboxylase and lipogenic transcription factor SREBP-1, possibly mediated by AMPK (Zhou et al., 2001). This induces fatty acid oxidation and suppresses expression of lipogenic enzymes (Zhou et al., 2001). In two randomized trials, significantly lowered alanine aminotransferase (ALT) levels were reported after treatment with the lipid-lowering and insulin-sensitizing drug metformin (Uygun et al., 2004). This improvement of aminotransferase levels was sustainable, which was assessed in 6- and 12-month follow-ups (Uygun et al., 2004).

By comparison, thiazolidinediones act as agonists of the nuclear receptor PPARγ and improve insulin sensitivity by diverse mechanisms, including stimulation of adipocytes to increase uptake of free fatty acids and release adipokine, thereby stimulating a redistribution from liver fat to peripheral tissue (Shulman, 2000; Yki-Järvinen, 2004). In clinical trials, both first- and second-generation thiazolidinediones were reported to decrease aminotransferase levels and improve histopathological disease stages (Reynaert et al., 2005, Promrat et al., 2004; Balas et al., 2007). In particular, second-generation thiazolidinediones pioglitazone and rosiglitazone achieved promising results in clinical trials (McCul-

lough, 2006b; Angelico et al., 2007). Treatment with pioglitazone and rosiglitazone for 4 and 6 months, respectively, improved insulin resistance, reduced hepatic lipid content, and lowered resistin plasma levels in patients with type 2 diabetes (Bajaj et al., 2004; Jung et al., 2005). Therapeutic benefit of thiazolidinedione treatment of NAFLD, however, should be critically evaluated, because discontinuation of treatment with either pioglitazone or rosiglitazone after 48 weeks abolished the therapeutic effects on NASH, resulting in increased liver fat, inflammatory activity, and aggravation of histopathological scores (Neuschwander-Tetri et al., 2003; Lutchman et al., 2007). Given the fact that long-term treatment would be required to suppress NAFLD, beneficial effects of thiazolidinedione treatment may ultimately be limited by treatment-associated weight gain (Lutchman et al., 2007; Ratziu et al., 2008).

In a recently reported randomized placebo-controlled study with 63 patients, about 50% of patients responded to rosiglitazone therapy with improved steatosis and transaminase levels despite an additional gain of 0.5 kg body weight compared with the placebo group (Ratziu et al., 2008). Most notably, in this particular study and in contrast to former reports (i.e., Sanyal et al., 2004), there was no improvement of histopathological parameter (Ratziu et al., 2008), which again seriously calls into question the significance of serum transaminases as surrogate markers for liver function in NAFLD. This notion is further supported by characterization of the molecular effects of thiazolidinedione in livers of patients with NASH that showed no improvement of organelle dysfunction, such as that evaluated by scoring of microsteatosis, cellular enlargement, dilated endoplasmic reticulum, or apoptosis as well as by determination of endoplasmic reticulum stress (Caldwell et al., 2007; Das et al., 2008). In addition, pioglitazone treatment could not protect cultures of hepatocytes and adipocytes from FA-induced ER stress in vitro (Das et al., 2008). On the contrary, results from transmission electron microscopy studies in liver biopsies of patients before and after treatment with rosiglitazone for 48 weeks revealed an increase in crystalline inclusions (from 4.0 to 7.2%) of unclear pathological relevance in liver mitochondria of patients with NAFLD (Caldwell et al., 2007). This suggests that insulin-sensitizing thiazolidinediones may suppress symptoms of NAFLD during treatment. Future studies under standardized conditions are required to explore the therapeutic benefit for a treatment with thiazolidinedione in combinational treatment courses as well as for certain patient cohorts (i.e., PPARγ positive NAFLD cases). Treatment of insulin resistance may be a therapeutic strategy in the treatment of NAFLD. However, there is certainly a need to discover less invasive but more reliable disease markers, which may replace diagnostic liver biopsies to provide prognostic information.

B. Lipid-Lowering Agents and Bile Acid Substitution in the Therapy of Nonalcoholic Hepatic Steatosis and Steatohepatitis

From clinical studies positive results were reported regarding the use of lipid-lowering agents, such as fibrates, statins, and probucol, in the treatment of NASH (Kadayifci et al., 2007). Fibrates are ligands of PPARα, thereby affecting lipid oxidation, lowering serum TAG levels, and increasing serum HDL by stimulating lipoprotein lipase and regulating apolipoprotein expression (Cook et al., 2000; Neve et al., 2000). Studies in animal models of steatosis and steatohepatitis, however, have demonstrated fibrates to ameliorate insulin resistance, stimulate β-oxidation, and prevent inflammation (Chou et al., 2002; Akbiyik et al., 2004; van Raalte et al., 2004; Haluzik et al., 2006; Nagasawa et al., 2006). The finding that bezafibrate inhibited hepatic stellate cell activation and fibrogenesis in a murine model of steatohepatitis, suggested that fibrates may be of putative value for the prevention of disease progression (Nakano et al., 2008). Treatment with clofibrate showed no positive effects on transaminase levels or histopathology indices, whereas gemfibrozil treatment lowered ALT levels after 4 weeks of treatment. (Laurin et al., 1996; Basaranoglu et al., 1999). The potent effects of fibrates on macrovesicular steatosis were reported in a study in which pretreatment of 11 obese living liver transplant donors with bezafibrate (400 mg/day) significantly improved hepatic steatosis (by up to 18%) and was able to normalize liver function and lipid metabolism (Nakamuta et al., 2005; Perkins, 2006). Improvement of biochemical parameters (ALT > aspartate aminotransferase, alkaline phosphatase, and GGT) was also reported after treatment with fenofibrate (200 mg/day) as assessed in 16 patients with biopsy-confirmed NAFLD (Fernández-Miranda et al., 2008). Note that the grade of hepatocellular ballooning degeneration was reduced after treatment for 48 weeks, but grade of steatosis, lobular inflammation, fibrosis, or NAFLD activity score was not significantly changed (Fernández-Miranda et al., 2008). One report noted a paradox increase in resistin levels after fenofibrate treatment, which otherwise is associated with an increase in insulin resistance (Haluzik et al., 2006). Likewise, treatment of patients with NAFLD with the lipid-lowering and antioxidant agent probucol was found to be effective in normalizing aminotransferase levels (Merat et al., 2003a,b) and improved steatohepatitis in regard to histopathological aspects (Merat and Malekzadeh, 2007; Merat et al., 2008).

Statins, such as atorvastatin, pravastatin, and rosuvastatin, lower serum lipid concentrations by inhibiting HMG-CoA reductase, the key enzyme in cholesterol biosynthesis, and have been evaluated in regard to their use for the treatment of NAFLD (Kiyici et al., 2003; Rallidis et al., 2004; Onofrei et al., 2008). In five independent studies, in which a total of 147 patients with

TABLE 1
Therapeutic strategies in the treatment of NAFLD

Therapeutic Strategy	Prototypic Agent	Putative Mechanism of Action	References
Weight loss	Orlistat	Pancreatic lipase inhibitor; inhibits gastrointestinal fat uptake, reduces lipid overload via reduction of plasma lipids	Zelber-Sagi et al., 2006; Hussein et al., 2007
Insulin sensitizing	Pioglitazone	PPARγ agonist; redistribution of hepatic lipids to peripheral organs, reduced intrahepatic fat	Bajaj et al., 2004; Promrat et al., 2004; Sanyal et al., 2004; Jung et al., 2005; Belfort et al., 2006; Reynaert et al., 2005; Balas et al., 2007; Lutchman et al., 2007; Merat and Malekzadeh, 2007; Ratziu et al., 2008
Lipid lowering	Atorvastatin	HMG-CoA reductase inhibitor; reduces serum cholesterol, reduces lipid overload via reduction of plasma lipids	Horlander et al., 1997; Kiyici et al., 2003; Hatzitolios et al., 2004; Antonopoulos et al., 2006; Athyros et al, 2006; Gómez-Domínguez et al., 2006
Antioxidant strategies	Vitamin E	ROS scavenger, reduces oxidative stress	Harrison et al., 2003a; Vajro et al., 2004; Loguercio et al., 2007; Yakaryilmaz et al., 2007; Machado et al., 2008
Antiapoptotic strategies	18β-Glycyrrhetinic Acid	Unknown mechanism, prevention of lipotoxic effects (lysosomal and mitochondrial pathways), in vitro and animal models	Wu et al., 2008
Antiinflammatory strategies	Infliximab	TNF-α antibodies; inhibition of TNF-α-mediated activation of leukocytes and effects on other cell types	Li et al., 2003; Barbuio et al., 2007; Koca et al., 2008
Antiplatelet strategies	Ticlopidine	ADP receptor inhibitor, inhibition of platelet aggregation	Fujita et al., 2008
Antifibrotic strategies	Losartan	Inhibition of AT II receptor, reduces stellate cell proliferation and collagen production	Yokohama et al, 2004; Georgescu and Georgescu, 2007
Others	Exenatide	Glucagon-like peptide (GLP-1) analog, stimulation of IR, reduction of intrahepatic fat	Tushuizen et al., 2006
	Dietary *n*-3 PUFAs	Activation of fatty acid oxidation, reduced De novo lipid synthesis, ROS scavenger, antilipotoxic effects (improvement of hepatic and peripheral insulin resistance)	Sekiya et al., 2003; Capanni et al., 2006; Le Foll et al., 2007; Allard et al., 2008; Machado et al., 2008

NAFLD received atorvastatin for 24 weeks and 21 months, an improvement of serum transaminases was reported for 59 to 78% of the cohort (for review, see Onofrei et al., 2008; for references, see Table 1). There is also evidence for histological improvement of NAFLD and sustained reduction in hepatic steatosis after treatment with statins (Horlander and Kwo, 1997; Rallidis et al., 2004; Ekstedt et al., 2007). Despite concerns regarding the safety of statins, their use for the treatment of NAFLD has been encouraged and considered safe by the National Lipid Association expert panel (Cohen et al., 2006). In particular, statins may have the potential to improve insulin sensitivity and have anti-inflammatory activity by mechanisms involving suppression of NF-κB activation and/or increasing iNOS mRNA stability as suggested from animal and in vitro studies (Ahn et al., 2007; Dombrecht et al., 2007; Habara et al., 2008; Lalli et al., 2008).

C. Other Approaches

Various antioxidant strategies have been explored for their use in the therapy of NAFLD, including radical scavenger vitamin E (α-tocopherol), β-carotene, and *n*-3 PUFAs, which are currently explored for their protective effect in NASH (Perlemuter et al., 2005; Kadayifci et al., 2007). Dietary supplementation with polyunsaturated fatty acids is thought to improve NAFLD by preventing lipid peroxidation, positively influencing peripheral insulin resistance, activating PPARα, and suppressing lipogenic transcription factor SREBP-1 (Keller et al., 1993; Sekiya et al., 2003; Gutiérrez et al., 2007; Le Foll et al., 2007). Studies in mice suggested that microcirculatory failure in macrosteatotic livers might be corrected by dietary supplementation of PUFAs through correction of reduced levels of *n*-3 and *n*-6 PUFAs in livers of patients with NAFLD (El-Badry et al., 2007; Allard et al., 2008). In a randomized, double-blind trial in 42 patients with NAFLD who received a 1-g capsule of PUFAs daily for 12 months, the treated patients displayed significantly reduced levels of serum TAG, liver transaminases, GGT, and improved steatosis (as assessed by ultrasonographic measures) (Capanni et al., 2006). The value for the dietary supplementation of PUFAs in NAFLD has been questioned by findings in a murine model of steatohepatitis, in which *n*-3 PUFAs failed to prevent the development of steatohepatitis because of accumulation of hepatic lipoperoxides (Larter et al., 2008a). Such effect, however, may be encountered by

combination with other antioxidative strategies, such as treatment with vitamin E. Data from clinical studies had indicated that dietary supplementation with vitamin E in patients with NASH is associated with significant effects on liver transaminase levels and histopathology of NASH, and it may improve serum TNF-α levels (Lavine, 2000; Hasegawa et al., 2001; Harrison et al., 2003a; Kugelmas et al., 2003; Sanyal et al., 2004; Dufour et al., 2006; Yakaryilmaz et al., 2007). A combination of vitamin E with phospholipids and silymarin, an extract of milk thistle seed (silybum marianum), resulted in an improvement of hepatic steatosis (as assessed by ultrasonographic scores) and hyperinsulinemia, lowered liver transaminase levels and other indices of liver fibrosis as well as plasma levels of TGF-β and TNF-α in patients with NAFLD (Loguercio et al., 2007). However, as concluded from a *meta*-analysis of six randomized trials to investigate antioxidant supplements for the treatment of NAFLD, the information currently available is not sufficient to draw conclusions on the benefits or risks of antioxidant supplements in the therapy of NAFLD (Miglio et al., 2000; Harrison et al., 2003a; Pamuk and Sonsuz, 2003; Vajro et al., 2004; Lirussi et al., 2007a). Therefore, further studies in larger patient cohorts are required to provide information on recommendable doses and possible risks associated with vitamin E supplementation in NAFLD (Guallar et al., 2005).

Other antioxidant and/or anti-inflammatory substances currently under investigation for their therapeutic effects in NASH are, silymarin, which may prevent stellate and Kupffer cell activation (via reduction of ROS and leukotriene B_4 synthesis) (Dehmlow et al., 1996; Di Sario et al., 2005) and PDE_4 inhibitor pentoxifylline, which decreases lipopolysaccharide-stimulated TNF-α production (Neuner et al., 1994; Adams et al., 2004). Pentoxifylline was demonstrated to decrease ALT and GGT levels after 10 weeks of treatment (400 mg b.i.d.) in patients with NAFLD (Georgescu and Georgescu, 2007). *N*-Acetylcysteine, *S*-adenosyl-L-methionine, taurine, and betaine have been evaluated based on the rationale of supporting GSH-mediated oxidative stress defense (Bruun et al., 2002; Oudit et al., 2004; Bastard et al., 2006; Oz et al., 2006; Simons et al., 2007). *N*-Acetylcysteine acts as a cysteine donor for biosynthesis of γ-glutamylcysteine, which again is a substrate of GSH synthetase and GSH biosynthesis. In contrast, *S*-adenosyl-L-methionine replenishes intracellular GSH and mitochondrial GSH by providing for generation of homocysteine and cysteine, (García-Ruiz et al., 1995; Colell et al., 1997, 1998), and demonstrated to exert antiapoptotic and anti-inflammatory effects by lowering production of TNF-α (Hevia et al., 2004; Veal et al., 2004; Song et al., 2005; Oz et al., 2006). Positive effects in patients with NASH were reported for all four agents (Osman et al., 1993; Abdelmalek et al., 2001; Lieber, 2002; Pamuk and Sonsuz, 2003).

Nutritional supplementation with the hydrophilic bile acid ursodeoxycholic acid (Lindor et al., 2004) is thought to exert cytoprotective effects on liver cells (Heller et al., 1990; Ouazzani-Chahdi et al., 2007) that are not completely understood but include 1) protection of cholangiocytes (Marzioni et al., 2006) and hepatocytes (Danchenko et al., 2001) against the cytotoxicity of hydrophobic bile acids, 2) stimulation of hepatobiliary secretion (Fiorotto et al., 2007), and 3) inhibition of liver cell apoptosis (Lirussi and Okolicsanyi, 1992; Rodrigues et al., 1998; Silva et al., 2001; Solá et al., 2004, 2006). So far, four randomized trials with 279 patients have been performed to evaluate the efficacy of ursodeoxycholic acid in the treatment of NAFLD (Santos et al., 2003; Lindor et al., 2004; Méndez-Sánchez et al., 2004; Ersöz et al., 2005). Although improvement in liver function parameters was reported in individual trials, a meta-analysis showed that ursodeoxycholic acid treatment was not associated with significant differences in mortality or liver function tests (Orlando et al., 2007). The data available thus far do not support a decision for or against the use of UCDA in the treatment of NAFLD.

Finally, antibiotics and probiotics are being investigated for their effects in NASH (Lirussi et al., 2007b), an approach based on the notion that bacterial overgrowth together with increased intestinal permeability may be associated with pro-inflammatory stimulation and elevated TNF-α levels in NAFLD (Wigg et al., 2001; Riordan et al., 2002). Although antibiotics [e.g., tetracycline may decrease inflammatory stimuli by reducing intestinal bacteria and subsequent production of TNF-α (Lichtman et al., 1991)], probiotics are bacteria that are defined by the positive effects they exert on their host's health and that are expected to normalize intestinal bacterial overgrowth in NAFLD (Lenoir-Wijnkoop et al., 2007). Studies in *ob/ob* mice emphasized the putative therapeutic advantage of probiotics in NAFLD, which normalized fatty acid oxidation in mouse livers, alleviated steatosis and reduced expression of UCP-2 as well as activity of JNK and NF-κB (Li et al., 2003). Probiotic VSL3 (a mixture of four lactobacilli strains) was found to improve plasma levels of malondialdehyde and 4-hydroxynonenal in a nonrandomized study with 22 patients with NAFLD, suggesting that the probiotic may contribute positive effects in a treatment strategy for NAFLD (Loguercio et al., 2005).

D. Novel Targets for Therapeutic Intervention in Nonalcoholic Hepatic Steatosis and Steatohepatitis

New treatment strategies focus on the molecular basis of critical events in the development or progression of NAFLD. Novel targets are 1) tyrosine kinases, pro-lipogenic enzymes and nuclear receptors related to impaired insulin signaling, 2) enhanced peripheral lipolysis, 3) elevation of free fatty acids, 4) reduced hepatic lipid oxidation, 5) FA-induced organelle toxicity, 6) release of pro-inflammatory cytokines, and 7) activation of stellate

cells (Fiorucci et al., 2007). A few of these strategies are addressed below. Phosphotyrosine kinases (e.g., PKC-θ, PKC-δ, PKC-β) and JNK1, which interfere with IRS signaling, may represent reasonable targets for the treatment of insulin resistance (Shulman, 2000; Samuel et al., 2007). Knock down of PKCε in animal studies successfully protected rats from fat-induced hepatic insulin resistance, which was reflected by an increase of IRS2 tyrosine phosphorylation (approximately 300% above basal levels) and an 8-fold increase in insulin-stimulated AKT2 activity compared with the control groups (Samuel et al., 2007). Another therapeutic strategy for the treatment of insulin resistance includes suppression of AMPK, which leads to activation of mammalian target of rapamycin and subsequent induction of raptor-dependent phosphorylation of IRS-1 on Ser636/639 and IRS-1 degradation (Harrington et al., 2004; Tzatsos and Kandror, 2006; Mordier and Iynedjian, 2007). Furthermore, association of PPARγ coactivator-1 with functional polymorphisms in patients with insulin resistance and reduced activity of PPARγ coactivator-1 in NAFLD livers led to the implication that its activation may have advantageous effects in NAFLD (Medina-Gomez et al., 2007; Westerbacka et al., 2007; Hui et al., 2008). Finally, lipid droplet-associated proteins have been suggested as putative targets for the pharmacological intervention in insulin resistance. This was at least suggested by the finding that lipid droplet fusion protein SNAP23 mediates membrane fusion of GLUT-4 vesicles in response to insulin (Kawanishi et al., 2000) and that transfection of cardiomyocytes with SNAP23 alleviated oleic acid-induced insulin resistance (Boström et al., 2007).

It is noteworthy that reduction of intrahepatic malonyl-CoA levels by targeting acetyl-CoA carboxylases 1 and 2 with oligonucleotides was reported to lower hepatic lipid contents (e.g., long-chain acyl-CoAs, diacylglycerol, and triacylglycerols) and improved hepatic insulin sensitivity in an animal model of fat-induced NAFLD (Yamaguchi et al., 2007). In contrast, targeting TAG accumulation in the liver by knockdown of DGAT isoforms 1 and 2 (the enzyme that catalyzes the final step in triglyceride synthesis) was a less successful approach to prevent NAFLD in rodent models (Yu et al., 2005; Choi et al., 2007; Yamaguchi et al., 2007). An initially positive effect on insulin resistance, plasma fatty acids, and diacylglycerol levels was annihilated by an increase in markers of oxidative stress and fibrosis (Choi et al., 2007; Yamaguchi et al., 2007). Supported by in vitro data that indicated TAGs may protect from toxic lipids and/or ROS, inhibition of triglyceride synthesis seems to be an unsuitable therapeutic target (Yamaguchi et al., 2007).

Likewise, the strategy to improve lipoprotein profiles, targeting peripheral lipolysis to reduce serum levels of free fatty acids seems to be a reasonable intervention in NAFLD. The positive influence of different drugs, such as metformin, thiazolidinediones, and salicylates, on NAFLD has been related, at least in part, to their modulation of peripheral lipolysis (Ren et al., 2006). In particular, thiazolidinediones and salicylates are thought to reduce TNF-α-induced lipolysis, the latter of which potentially involves a mechanism that prevents reduction of perilipin A (Souza et al., 1998; Zu et al., 2008). Furthermore, salicylates are thought to prevent inflammation by inhibition of IKK-β and NF-κB and were recently suggested for the treatment of insulin resistance and type 2 diabetes (Chen, 2005; Lappas et al., 2005; Möhlig et al., 2006). In adipocytes, aspirin was shown to inhibit TNF-α-mediated phosphorylation of IRS-1 at Ser307 as well as the phosphorylation of JNK, c-Jun, and degradation of IKK-β (Gao et al., 2003). In particular, inhibition of Kupffer cell activation, COX-2, and LO-5 may be further explored experimentally for the treatment of early disease stages, although interference with regenerative pathways as well as the pro-/antiapoptotic balance may bare the risk of disease aggravation (Bykov et al., 2006; Horrillo et al., 2007; Stafford and Marnett, 2008). Furthermore, a possible role for anti-inflammatory agents in the prevention of lipotoxic cell death was suggested by the finding that phospholipase A_2 inhibitors were able to block lipotoxic cell death induced by palmitate possibly mediated by reduction of LPC (Han et al., 2008). Likewise, inhibition of Foxo1, which was found to protect against FA-induced apoptosis and ER stress, was proposed as a novel therapeutic strategy in NAFLD that remains to be evaluated (Martinez et al., 2008).

The renin-angiotensin-aldosterone system was proposed as pharmaceutical target in NASH to prevent stellate cell activation and fibrotic remodeling of the liver (Warner et al., 2007). In addition, in animal models of NASH, the angiotensin II type I receptor antagonist telmisartan reduced (more effectively than valsartan) hepatic triglyceride contents dose dependently, improved ALT and TNF-α levels, and suppressed fibrosis (Fujita et al., 2007). These advantageous effects of telmisartan on hepatic lipid storage were explained by its acting as a PPARγ agonist to reduce hepatic steatosis, in addition to its ability to block the renin-angiotensin pathway (Benson et al., 2004; Schupp et al., 2006; Fujita et al., 2007). Losartan was reported to improve clinical chemistry parameters of liver injury (Yokohama et al., 2004). Before lipid-lowering, hepatoprotective, and antioxidant strategies, losartan efficiently reduced hepatic steatosis, liver transaminase levels, and necroinflammation in 12 patients with dyslipidemia- and hypertension-associated NASH (Georgescu and Georgescu, 2007).

Remodeling processes during NASH are also experimentally approached by targeting nuclear receptors, such as the farnesoid X receptor (FXR), LXR and PPARγ, which are involved in regulation of hepatic stellate cells (Marra et al., 2000; Wright, 2006). In particular, activation of FXR was found to protect from liver

injury in rodent models and was shown to suppress trans-differentiation of stellate cells in vitro (Fiorucci et al., 2004, 2005a,b). Likewise, PPARγ agonists such as troglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 may have antifibrotic effects in vivo, because they prevented proliferation of human hepatic stellate cell in vitro possibly by a mechanism antagonizing TGFβ1/Smad3-signaling (Marra et al., 2000; Zhao et al., 2006). For further evaluation of therapeutic approaches involving PPAR agonists, it should be considered that administration of PPARα and PPARγ agonists (e.g., WY-14643 and pioglitazone) was linked to an accumulation of ceramide, which is thought to be a mediator of lipotoxicity in the heart (Zendzian-Piotrowska et al., 2006; Baranowski et al., 2007). Under conditions of lipid burdening, treatment with PPAR agonists may therefore be associated with a risk to enhance sensitivity to lipotoxicity in the heart and/or liver (Listenberger and Schaffer, 2002; Ghosh and Rodrigues, 2006).

Further research should specifically address signaling pathways that trigger cellular checkpoints in the determination of reversibility/progression of hepatic steatosis (Yin et al., 2006; Lee et al., 2007). In addition, lipid droplet-associated proteins and lipid mediators in the inflammatory cross-talk of liver cells may provide promising targets in the therapy of NAFLD.

IV. Conclusion

The development of hepatic steatosis is a multifactorial process that ultimately leads to impairment of lipid processing and clearance in the liver. Insulin resistance is though to be a major component for the development of NAFLD. Lipotoxic and inflammation-mediated mechanisms have been suggested to be responsible for adipocyte dysfunction and modulation of peripheral lipid storage capacities, which result in release of free fatty acids and hepatic lipid burdening.

In NAFLD, the liver fails to cope with an overflow of lipids. Lipotoxic effects of free fatty acids and lipid intermediates impair proper function of liver cell organelles by mechanisms that are not yet completely understood, but involve production of ROS, ER stress, activation of proinflammatory defense programs, and eventually apoptosis. Toxic lipids and release of cytokines foster insulin resistance by activating JNK, PKCζ, PKCε, and other phosphokinases to impair IRS-1 and IRS-2 signaling. This disturbed insulin signaling contributes to diminished fatty acid oxidation as well as VLDL assembly and secretion in the liver, involving an inadequate regulation of PPARα and PPARγ and failure to properly inactivate SREBP-1 and ChREB (Ip et al., 2003; Browning et al, 2004; Reddy and Rao, 2006). Dysregulation in the activity of FOXO1 and FOXA2 and of nuclear receptors, such as HNF4α, nuclear factor-Y, LXR, RXR, and possibly CAR and PXR, are important events in steatosis and steatohepatitis and provide a molecular rational for the deteriorating metabolic dysfunction in NAFLD (George and Liddle, 2008). Several mechanisms responsible for reduced lipid combustion and elevated de novo synthesis of lipids resulting in hepatic steatosis, however, may run in parallel and need to be delineated along the lines of insulin resistance and lipotoxicity.

The progression of hepatic steatosis is determined by collaborative events but is far from clear. Induction of PPARγ and lipid droplet-associated proteins in the liver enable formation of lipid droplets that incorporate various lipids and provide storage for de novo synthesized triglycerides. These lipid droplets, up to a certain point, may protect hepatocellular organelles from toxic lipids and ROS, which are produced from elevated fatty acid oxidation, among other sources. In the course of metabolic overload, the hepatocellular defense against oxidative stress is challenged by lipotoxic effects, deteriorating organelle dysfunction, and enlargement of steatotic hepatocytes, which may impair proper microcirculation. It remains uncertain, whether lipotoxicity alone (and which lipid classes in particular) may sufficiently explain organelle toxicity in vivo and whether there is a chronological order of organelle dysfunction. Observations in vitro and in vivo are suggestive for mitochondria to be the first organelle to be damaged, as judged by decreased mitochondrial fatty acid oxidation, compensatory increased peroxisomal fatty acid oxidation, and presence of ultrastructural altered mitochondria (Begriche et al., 2006). This possibly provokes damage in other organelles as well through excessive production of ROS. Adaptive processes to long-term exposure with oxidative stress were suggested to further increase the vulnerability of hepatocytes to toxic stimuli and stress (Diehl, 2005). Recent findings have highlighted the role of ER stress early in hepatic steatosis, which may crucially alter transport of nuclear receptors and maintenance of lipid trafficking (Kaplowitz et al., 2007; Yang et al., 2007).

Activation of cellular defense programs, specifically activation of NF-κB, seems to be a major determinant for disease progression from steatosis to steatohepatitis, entailing inflammation as well as insulin resistance (Cai et al., 2005). Although activation of NF-κB may be hepatoprotective, its activation of other liver cell populations (e.g., endothelial cell and Kupffer cells) could trigger intercellular cascades to induce and maintain inflammation. Such activation of Kupffer cells provides additional stress stimuli (TNF-α, ROS, IL-6, PGE_2) and may shift the cellular fate of hepatocytes from survival toward apoptosis by altering lipid oxidation and the intracellular redox state. The latter was found to be a critical factor for the pro- and/or antiapoptotic effects of NF-κB and TNF-α to prevail (Nobili et al., 2005; Garcia-Ruiz and Fernandez-Checa, 2006). Furthermore, activation of JNK-related signaling has been suggested to be at least one critical step in promoting progression from triglyc-

eride accumulation and steatosis to inflammation, lipid peroxidation, and liver injury associated with steatohepatitis (Schattenberg et al., 2006; Lu and Archer, 2007).

The event or cell type that provides the signal that eventually results in the cross-talk of liver cells that maintains the inflammatory environment in NAFLD remains to be determined. Activation of Kupffer cells may be initiated by leukocytes, but so far, only pieces of the puzzle of intercellular communication leading to this event have been discovered. The same is true for the incidents resulting in stellate cell transactivation and fibrotic remodeling. Characterization of the role of endocrine factors (e.g., adipokines, ATII, noradrenalin, and recently brought up cannabinoid receptors) and altered availability and intercellular processing of lipids for intercellular communication of liver cells in NAFLD seem to be of critical importance for an understanding of disease progression and the development of novel therapeutic approaches. In this regard, very recent experimental evidence is suggestive for a role of hedgehog signaling in hepatic stellate cell activation, whereby sonic hedgehog acts as an autocrine viability factor for myofibroblastic hepatic stellate cells (Sicklick et al., 2005; Fleig et al., 2007; Yang et al., 2008). Finally, furthering an understanding of molecular processes in lipid droplet-associated distribution and utilization of cellular lipids as well as of signaling molecules (i.e., via lipid rafts and RTKs) may help to explain the observed alterations of LDs in insulin resistance and to evaluate their relevance for the pathogenesis of NAFLD. From the current point of view, strategies for the long-term reduction of intrahepatic lipid storage and free fatty acid levels seem to be most promising for the prognosis of NAFLD. In particular, positive effects of weight loss may most effectively prevent NAFLD by encountering its major risk factors, such as dyslipidaemia and insulin resistance, among others. Future research carries the hope to unravel the signaling pathways associated with early (reversible) and late (irreversible) stages of steatosis to provide novel therapeutic targets in NAFLD progression. For the evaluation of pharmacological therapies and management of disease, however, there is an immediate and urgent need to search for reliable markers of disease activity and progression.

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