Int. J. Exp. Path. (2006), 87, 1–16

CURRENT STATUS REVIEW

Mouse models in non-alcoholic fatty liver disease and steatohepatitis research

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INTERNATIONAL JOURNAL OF EXPERIMENTAL PATHOLOGY

Summary

Non-alcoholic fatty liver disease (NAFLD) represents a histological spectrum of liver disease associated with obesity, diabetes and insulin resistance that extends from isolated steatosis to steatohepatitis and cirrhosis. As well as being a potential cause of progressive liver disease in its own right, steatosis has been shown to be an important cofactor in the pathogenesis of many other liver diseases. Animal models of NAFLD may be divided into two broad categories: those caused by genetic mutation and those with an acquired phenotype produced by dietary or pharmacological manipulation. The literature contains numerous different mouse models that exhibit histological evidence of hepatic steatosis or, more variably, steatohepatitis; however, few replicate the entire human phenotype. The genetic leptin-deficient (*ob/ob*) or leptin-resistant (*db/db*) mouse and the dietary methionine/choline-deficient model are used in the majority of published research. More recently, targeted gene disruption and the use of supra-nutritional diets to induce NAFLD have gained greater prominence as researchers have attempted to bridge the phenotype gap between the available models and the human disease. Using the physiological processes that underlie the pathogenesis and progression of NAFLD as a framework, we review the literature describing currently available mouse models of NAFLD, highlight the strengths and weaknesses of established models and describe the key findings that have furthered the understanding of disease pathogenesis.

Keywords
animal, diabetes, leptin, review, steatohepatitis

Received for publication:
03 November 2005
Accepted for publication:
08 November 2005

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Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a spectrum of liver disease encompassing steatosis (fatty change), non-alcoholic steatohepatitis (NASH) and cirrhosis in the absence of alcohol abuse. NASH has been reported worldwide and is increasingly recognized as the leading cause for liver dysfunction and cirrhosis in the non-alcoholic, viral hepatitis negative population in Europe and North America (Skelly *et al.* 2001; Angulo & Lindor 2002). Population studies show that NAFLD is strongly associated with obesity

(Ludwig *et al*. 1980; Powell *et al*. 1990; Wanless & Lentz 1990; Ratziu *et al*. 2000), dyslipidaemia (Powell *et al*. 1990; Sanyal 2002), insulin resistance (IR) (Powell *et al*. 1990; Marchesini *et al*. 1999; Sanyal 2002) and type II (non-insulin dependent) diabetes mellitus (Powell *et al*. 1990; Sanyal 2002). Most authors now consider NAFLD to be a hepatic manifestation of the metabolic syndrome (Sanyal 2002; Marchesini *et al*. 2003).

Steatosis represents both an important cofactor that may determine of rate of progression of fibrosis in a range of liver diseases and a pathogenic process in its own right. The rate of progression of many liver diseases is accelerated when there are multiple coincidental insults [e.g. alcohol intake and chronic viral hepatitis (Poynard *et al*. 1997) or haemochromatosis (Fletcher *et al*. 2003)]. Similarly, the extent of hepatic steatosis has been shown to interact with several aetiological factors including chronic hepatitis C (Hourigan *et al*. 1999; Adinolfi *et al*. 2001; Hu *et al*. 2004) and alcohol (Teli *et al*. 1995; Reeves *et al*. 1996) as well as influencing the progression of lone steatohepatitis (Matteoni *et al*. 1999). Historically, lone hepatic NASH was considered a relatively benign disease in the majority of patients, however, currently available data suggest that NASH is progressive, leading to significant morbidity and may be a major cause of what was previously described as 'cryptogenic cirrhosis' (Caldwell *et al*. 1999; Poonawala *et al*. 2000; Reid 2001).

The development of NAFLD is determined by the interaction of genetic and environmental factors (Day 2002). Complex, polygenic disease traits, like NAFLD and IR, are now the focus of much research. Such diseases are difficult to study in humans due to genetic heterogeneity within populations, epistasis, gene–environment interaction and the low frequency of familial cases of diseases compared with the high overall frequency in the background population (Dragani 1998). Sharing many physiological, anatomical and metabolic similarities with the human, the laboratory mouse, *Mus musculus*, has been widely adopted as the primary model organism for research in this field. The many standardized and well-characterized inbred strains allow confounding factors such as genetic heterogeneity, gender, dietary or environmental variation and age to be eliminated as variables (Silver 1995). This allows researchers to study the effects of an intervention on a single homogeneous population.

Despite recent advances in elucidating the genetic contribution and pathogenesis of related conditions, such as IR and type II diabetes mellitus (Kahn 1994; Almind *et al*. 2001), our understanding of the pathogenesis of steatosis and steatohepatitis remains incomplete (Koteish & Mae 2002). The use of animal models in this field has proved invaluable. Indeed, it was work-describing inducible steatohepatitis in an animal model that led Day and James to propose the 'two-hit' hypothesis for the pathogenesis of NASH which remains a foundation for research in this field (Day & James 1998). The first hit, steatosis, sensitizes the liver to the induction of inflammation by a second pathogenic insult that promotes oxidative stress and hence steatohepatitis (Figure 1). This model has subsequently been revised in recognition that a combination of 'second hits' (both environmental and genetic) may lead to the development of steatohepatitis (Day 2002).

The pathogenesis of NAFLD

Steatosis and IR

The liver plays a central role in whole body lipid and carbohydrate metabolism (shown in Figure 2). Disruption of the normal mechanisms for synthesis, transport and removal of long-chain fatty acids (LCFA) and triglycerides (TG) are the basis for the development of NAFLD and provide a scheme for reviewing the available animal models. The genesis of steatosis is closely related to the development of obesity and particularly IR, a near universal finding in patients with NAFLD (Day 2002). Indeed, IR is associated with NASH even in subjects with apparently normal glucose tolerance (Marchesini *et al*. 1999). Steatosis occurs when the rate of import or synthesis of fatty acids by hepatocytes exceeds the rate of export or catabolism (Koteish & Diehl 2001; Bradbury & Berk 2004). The accumulation of lipid within the liver may be produced by disrupting the normal physiological balance in one of four main ways:

Increased delivery and uptake into hepatocytes of LCFA due to excess dietary intake or release of from adipose tissue stores. Adipose tissue is metabolically active and releases many biologically active products including mediators of carbohydrate metabolism (leptin, adiponectin and resistin), lipid metabolism (apolipoprotein E and lipoprotein lipase) and adipocytokines (TNFα, IL-6 and TGFβ) (Brunt 2004). As obesity develops, adipose TNFα expression has been shown to rise in patients and experimental models. This acts both in an autocrine and paracrine fashion activating IκB kinase β (Ικκβ) and inhibiting phosphorylation of insulin receptor substrates (IRS-1 and IRS-2). This in turn leads to failure of insulin-mediated suppression of hormone-sensitive lipase (HSL) and increased release of LCFA into the circulation (Day 2002; Shoelson *et al*. 2003). An explanation for the strong association between central obesity, characterized by predominant fat deposition in omental and mesenteric stores, and steatosis is that high concentrations of LCFAs are released directly into the portal circulation.

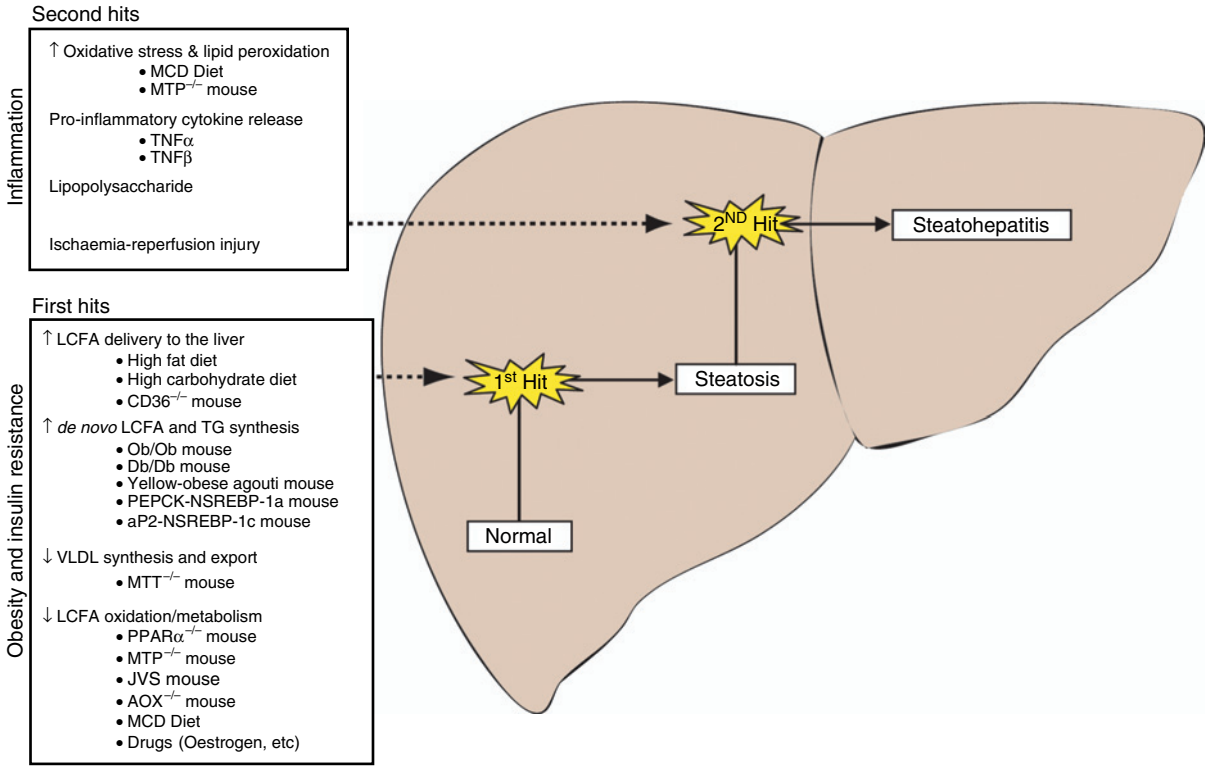

Figure 1 The 'two-hit' hypothesis for the pathogenesis on non-alcoholic fatty liver disease. The progression from normal healthy liver to steatohepatitis is in a stepwise fashion involving first the development of obesity and insulin resistance (which leads to fatty change) and later hepatic inflammation. Some of the available models and pathogenic processes are also summarized.

This, coupled with TNFα-mediated upregulation of hepatic fatty acid translocase (Memon *et al.* 1998), leads to enhanced uptake by the liver. Studies in mouse models have shown that disruption of the type 1 TNFα receptor gene (Uysal *et al.* 1997), the *Iκκβ* gene, or administration of salicylates inhibits *Iκκβ* activity (Yuan *et al.* 2001) and ameliorates IR. However, there remains debate as to whether steatosis also abates (Koteish & Mae 2002).

Increased de novo hepatic LCFA and TG synthesis. Accumulation of excess oleic acid (a product of *de novo* fatty acid synthesis) has been demonstrated in both humans and murine models of steatosis (Shimomura *et al.* 1998a; Araya *et al.* 2004). Sterol regulatory element-binding protein-1c (SREBP-1c) is a key membrane-bound transcription factor through which insulin promotes hepatic lipid synthesis (Table 1). Similarly, rising glucose levels activate *de novo* hepatic lipid synthesis via carbohydrate response element-binding protein (ChREBP) (Dentin *et al.* 2005). High plasma glucose and insulin levels following a carbohydrate meal normally trigger rapid synthesis of LCFAs within hepatocytes. Elevated insulin levels also promote GLUT4-mediated uptake of plasma glucose by adipocytes, synthesis of lipid stores and inhibition of lipolysis in adipose tissue.

In obese individuals, there is less GLUT4 activity leading to greater postprandial hyperglycaemia, and further pancreatic insulin release (Carvalho *et al.* 2001). This, together with the increased release of TNFα from adipose tissue and consequent IR, drives further hepatic LCFA synthesis from acetyl CoA. The conversion of LCFAs to TGs by glycerol phosphate acyl transferase within hepatocytes is non-rate limiting, consequently increased availability of LCFAs will lead to increased hepatocyte TG synthesis and accumulation (Bradbury & Berk 2004).

Failure of very low-density lipoprotein synthesis and TG export. Hepatic clearance of LCFAs may only be accomplished by esterification to TG and export as very low-density lipoprotein (VLDL) or by oxidation. The transfer of lipid to apolipoprotein B100 by microsomal TG transfer protein (MTT) within the endoplasmic reticulum is the rate-limiting step in VLDL lipoprotein assembly. Studies in man have already demonstrated that mutations in the gene

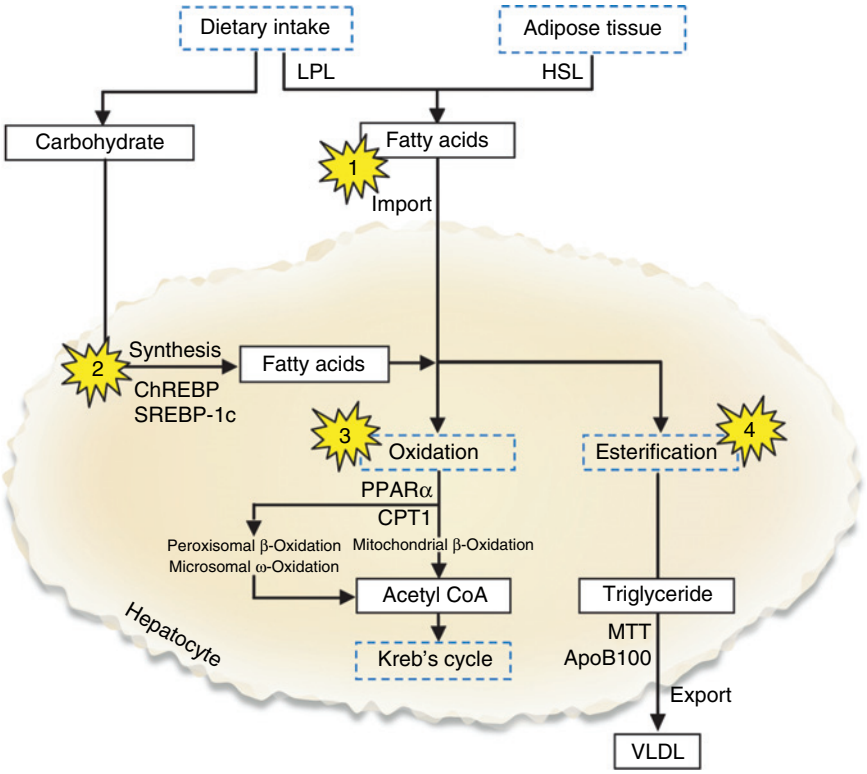

Figure 2 Hepatic lipid metabolism and the development of steatosis. In the absorptive state, dietary triglycerides (TG) are transported in the circulation as chylomicrons, LPL-mediated degradation releases LCFA. In the postabsorptive (fasting) state, insulin levels fall, and HSL releases LCFA from adipose tissue. In addition to absorbing circulating LCFA, hepatocytes synthesize LCFA from dietary carbohydrate under control of SREBP-1c and ChREBP. Hepatic clearance of fatty acids depends on oxidation in the mitochondria and peroxisomes (β-oxidation) or microsomes (ω-oxidation) to generate acetyl CoA for entry into Krebs cycle. Excess fatty acids are re-esterified to TG for export into the circulation as VLDL. Points at which disruption may produce non-alcoholic fatty liver disease are highlighted and discussed in the text. ApoB100, apolipoprotein B100; ChREBP, carbohydrate response element-binding protein; CPT1, carnitine palmonitoyl transferase 1; HSL, hormone sensitive lipase; LCFA, long-chain fatty acids; LPL, lipoprotein lipase; MTT, microsomal TG transfer protein; PPARα, peroxisome proliferator-activated receptor a; SREBP-1c, sterol regulatory element-binding protein 1c; VLDL, very low density lipoprotein.

encoding this enzyme are responsible for the autosomal-recessive condition abetalipoproteinaemia that is characterized by severe hepatic steatosis which may lead to cirrhosis (Wetterau *et al.* 1992; Berriot-Varoqueaux *et al.* 2000). Several commonly used drugs (including amiodarone, doxycycline and tetracycline) are known to affect β-oxidation and have also been shown to inhibit MTT activity in animal models of drug-induced steatosis (Letteron *et al.* 2003; Bradbury & Berk 2004).

Failure of LCFA elimination due to impaired hepatic mitochondrial β-oxidation. Oxidation of fatty acids takes place in three cellular organelles: mitochondria, peroxisomes and endoplasmic reticulum (microsomes) (Rao & Reddy 2001). Mitochondrial β-oxidation is the main route for the metabolism of short, medium and long-chain fatty acids under normal physiological conditions (Reddy 2001). This process may be disrupted at several key enzymatic stages.

Carnitine palmonitoyl transferase 1 (CPT1)-mediated trans-esterification and import of fatty acids into the mitochondrial matrix is the key rate-limiting and regulatory step in this process. CPT1 is inhibited by malonyl CoA, the first intermediate in fatty acid synthesis, and so is sensitive to the effects of upregulated hepatic fatty acid synthesis (McGarry *et al.* 1977; McGarry 2001). If CPT1, and hence the oxidative capacity of the mitochondria, become overwhelmed, alternative oxidative pathways in other subcellular organelles process a greater proportion of the LCFA load.

Once inside the mitochondrial matrix, acyl-CoA fatty acids are degraded by four sequential enzymatic reactions to release NADH, $FADH_2$ and acetyl-CoA (which then enters the tricarboxylic cycle). Mitochondrial trifunctional protein is a

Table 1 Selected transcription factors implicated in the pathogenesis of non-alcoholic fatty liver disease

Transcription factor	Endogenous agonist	Exogenous agonist	Target genes	Effects
Peroxisome proliferator-activated receptor α (PPARα, NR1C1)	Fatty acids, oxidized phospholipids, leukotrienes (LTB_4)	Fenofibrate, clofibrate, Wy-14643 (pirinixic acid)	Acyl-CoA synthase, fatty acid transporter 1, CPT1, ABCA1	↑ Fatty acid uptake, activation to acyl-CoA and mitochondrial β-oxidation.
Peroxisome proliferator-activated receptor γ (PPARγ, NR1C3)	Fatty acids, prostaglandin J_2	Thiazolidinediones (e.g. rosiglitazone, pioglitazone, troglitazone)	Lipoprotein lipase, fatty acid transporter 1, acyl-CoA synthase	Adipocyte differentiation. ↑ LPL mediated fatty acid release from chylomicrons. ↑ Adipose tissue fatty acid uptake and lipogenesis. ↑ Adiponectin production
Sterol regulatory element-binding protein 1a (SREBP-1a)	Insulin and sterol depletion lead to ↑ transcription and activation		HMG CoA synthase, HMG CoA reductase, fatty acid synthase	↑ Cholesterol Synthesis and ↑ fatty acid/triglyceride synthesis
Sterol regulatory element-binding protein 1c (SREBP-1c)	Insulin and LXRα activity lead to ↑ transcription and activation		Glucokinase, acetyl CoA synthase and carboxylase, fatty acid synthase, ATP citrate lyase	↑ Fatty acid/triglyceride synthesis; ↑ Lipogenesis
Sterol regulatory element-binding protein 2 (SREBP-2)	Insulin and sterol depletion lead to ↑ transcription and activation		HMG CoA synthase, HMG CoA reductase, LDL receptor	↑ Cholesterol synthesis
Carbohydrate response element-binding protein (ChREBP)	Glucose		Liver pyruvate kinase, fatty acid synthase, acetyl CoA carboxylase	↑ Lipogenesis
Liver X receptor α (LXRα, NR1H3)	Cholesterol derived oxysterols and LCFAs	T0901317, GW3965	CYP7A1, ABCA1, ABCG1, SREBP-1c, liproprotein lipase, CETP, ApoE	↑ Fatty acid synthesis, ↑ bile acid synthesis, ↑ lipoprotein assembly

hetero-octamer composed of four α and four β subunits associated with the inner mitochondrial membrane that catalyses the final three of these reactions (Ibdah *et al*. 2001). In man, autosomal recessive inherited mutations in mitochondrial trifunctional protein (MTP) are associated with a steatotic Reye–like syndrome, cardiomypoathy or sudden death. There is also evidence that foetal carriage of MTP mutations is associated with HELLP syndrome and acute fatty liver of pregnancy (Ibdah *et al*. 2001).

Steatosis to steatohepatitis

The two-hit hypothesis dictates that, once steatosis has developed, the liver is 'sensitized', and so an inflammatory response may be precipitated by a variety of stimuli. As is the case in the histologically similar condition alcoholic steatohepatitis, two mechanisms are thought to be pivotal to the genesis of NASH:

Oxidative stress, lipid peroxidation and cell death. Oxidative stress has been implicated as an aetiological factor in many progressive liver diseases including alcoholic steatohepatitis, Wilson's disease, hepatitis C and exposure to toxins such as carbon tetrachloride (McClain *et al*. 2004). The presence of biological markers of oxidative stress has been demonstrated in both patients and animal models of steatohepatitis (Sanyal *et al*. 2001; George *et al*. 2003).

Oxidation of fatty acids within hepatocytes is a major source of reactive oxygen species (ROS) including singlet oxygen molecules, hydrogen peroxide, hydroxyl radicles and superoxide anions. As described above, mitochondrial β-oxidation is the main route for the metabolism of short, medium and long-chain fatty acids under normal physiological conditions (Reddy 2001). Within the mitochondria, the electron transport chain safely dissipates the majority of electrons released; some react to form superoxide anions and other ROS. The enzymes that mediate mitochondrial β-oxidation are regulated by peroxisome-proliferator-activated receptor α (PPARα), a member of the nuclear receptor subfamily of ligand-activated transcription factors; however, PPARα preferentially induces extra-mitochondrial β-oxidation and ω-oxidation pathways (Aoyama *et al*. 1998; Rao & Reddy 2001). As mitochondrial oxidative capacity becomes overwhelmed, alternative pathways in the peroxisomes (β-oxidation) and endoplasmic reticulum (cytochrome P450 enzyme -4A and -2E1-catalysed ω-oxidation) assume a greater role in hepatic fatty acid oxidation. IR further enhances CYP2E1-mediated ω-oxidation. Peroxisomal β-oxidation is not linked to the electron transport chain and so generates hydrogen peroxide. Similarly, ω-oxidation produces dicarboxylic acid and other sources of ROS that further contribute to cellular oxidative stress (Reddy 2001). At times of hepatic fatty acid overload and mitochondrial dysfunction (e.g. diabetes mellitus and obesity), these physiologically minor pathways increase the hepatocyte ROS load.

When the production of ROS exceeds the antioxidant capacity of the cell, cellular macromolecules are damaged. This includes damage to nuclear and mitochondrial DNA, phospholipid membrane disruption by lipid peroxidation and the release of proinflammatory cytokines (Pessayre *et al*. 2001; Browning & Horton 2004). The production of oxidative stress is further amplified by mitochondrial damage that induces loss of cytochrome c which disables the electron transport chain (Pessayre *et al*. 2002; McClain *et al*. 2004). Several different mouse models of steatohepatitis (including methionine/choline-deficient model (MCD) and *ob/ob* mice) are characterized by increased ROS production, mitochondrial DNA damage (as assessed by mitochondrial 8-hydroxy-2′-deoxyguanosine levels) and reduced expression of the DNA mismatch repair enzyme MutY (Gao *et al*. 2004). Lipid peroxidation of polyunsaturated fatty acids generates toxic aldehyde by-products including malondyaldehyde and hydroxynonenal that are more persistent than ROS and so damage more distant intracellular organelles and may cause cell death. These products directly activate fibrogenic hepatic stellate cells and are chemotactic for neutrophils, recruiting immunologically active cells into the inflammatory process (George *et al*. 2003). ROS may also induce Fas ligand expression on hepatocytes and promote paracrine-induced apoptotic cell death (Pessayre *et al*. 2001).

Pro-inflammatory cytokine-mediated hepatocyte injury. Many of the proinflammatory cytokine abnormalities that were first described in alcoholic liver disease have now been reported in NASH (Tilg & Diehl 2000). The association between TNFα expression and IR has already been discussed. Studies also demonstrate greater expression of TNFα and TNFα type 1 receptor in patients with steatohepatitis than those with steatosis alone (Crespo *et al*. 2001; Kugelmas *et al*. 2003). In addition, products of lipid peroxidation (e.g. 4-hydroxynonenal) have been shown to increase TGF-β1 expression, promoting hepatic fibrogenesis.

Animal models of NAFLD

The study of existing animal models (summarized in Table 2) has provided vital insights into the pathogenesis of steatosis and steatohepatitis, but these remain incompletely understood (Koteish & Mae 2002). The literature contains numerous different rodent models that exhibit histological evidence of

Table 2 Current mouse models of non-alcoholic fatty liver disease

Model	Description	DM/IR	Obese	Leptin	Fibrosis
Genetic: increased lipid import/synthesis					
ob/ob mouse	Leptin deficient. Steatosis may progress to NASH after 'second hit'.	Y	Y	↓	N
db/db mouse	Leptin receptor deficient. Steatosis may progress to NASH after 'second hit'.	Y	Y	↑	?*
Yellow-obese agouti (Ay) mouse	Hyperphagic: no hypothalamic appetite suppression by melanocortin.	Y	Y	–	N
$CD36^{-/-}$ mouse	Reduced peripheral lipid uptake increases fatty acid delivery to liver. Steatosis.	N	N	–	N
PEPCK-NSREBP-1a mouse	Lipoatrophic model. Hepatic SREBP-1a overexpression. Develops steatosis.	Y	N	↓	N
aP2-NSREBP-1c mouse	Lipoatrophic model. SREBP-1c overexpression in adipose tissue. Steatosis.	Y	N	↓	N
aP2-Diptheria toxin mouse	Lipoatrophic model. Transgenic expression of diptheria toxin in WAT.	Y	N	↓	N
Genetic: reduced lipid export/catabolism					
$PPAR\alpha^{-/-}$ mouse	Impaired mitochondrial β-oxidation. Develops steatosis.	N	N	–	N
Acetyl CoA $oxidase^{-/-}$ mouse	Defective β-oxidation leads to transient steatosis.	N	N	–	N
Aromatase (Cyp 19)-deficient mouse	Female mice lack oestrogen. Develops steatosis.	N	Y	–	N
$MTP^{-/-}$	Key enzyme for mitochondrial β-oxidation deleted. Develops steatohepatitis.	Y	?	?	?
Juvenile visceral steatosis mouse	Carnitine deficiency ↓ fatty acid transport into mitochondria for β-oxidation.	N	N	–	N
Environmental: increased lipid import/synthesis					
High fat diet	Increased adiposity, fatty acid and insulin resistance. Steatosis and NASH.	Y	Y	↑	?
High sucrose/fructose diet	Hepatic enzyme induction and increased fatty acid synthesis. Steatosis.	Y	Y	↑	?
Argenine deficient	Increased lipid synthesis. Steatosis due to abnormal orotic acid metabolism.	N	N	–	N
Environmental: reduced lipid export/catabolism					
Methionine/choline-deficient diet	Impaired mitochondrial β-oxidation. Develops steatosis, NASH and fibrosis.	N	N	–	Y
Steroids, oestrogen, tamoxifen	Impaired mitochondrial β-oxidation and reduced hepatic triglyceride secretion.	Y	Y	–	–

*Fibrosis may be reduced in the db/db mouse model.

hepatic steatosis. The features of true steatohepatitis that should include ballooning hepatocyte degeneration in addition to simply fatty change and an inflammatory infiltrate (Kleiner *et al.* 2005), are less frequently demonstrated in these models. No existing model exhibits the entire NAFLD phenotype as encountered in clinical practice, and many differ from the human disease in all but gross histological appearance. These inconsistencies and the lack of a reliable model of progressive fibrosing steatohepatitis have hampered research in this field.

Research models of NAFLD may be divided into two broad categories, those caused by either spontaneous or induced genetic mutation and those with an acquired NAFLD phenotype. The latter group may be produced by either dietary or pharmacological manipulation. The bulk of published research has employed the genetic leptin-deficient (*ob/ob*) or leptin-resistant (*db/db*) mouse and the dietary MCD model. However, these models differ significantly from the human phenotype in a number of pathogenically important ways and have led some to question whether observations made using many of these models are truly applicable to man. In this article, we will highlight the strengths and weaknesses of established models as well as summarizing those models that have been described since an earlier review (Koteish & Diehl 2001).

A model of genetically determined leptin deficiency (the ob/ob mouse)

The *ob/ob* mouse carries a spontaneous mutation first observed whilst intercrossing inbred mouse strains (Ingalls *et al.* 1950). This autosomal recessive trait renders animals hyperphagic, inactive, obese and severely diabetic with marked hyperglycaemia (Mayer *et al.* 1951a, b). Histological features include enlarged islets of Langerhans (consistent with compensatory hyperplasia due to IR) and hepatic fat deposition (Bleisch *et al.* 1952). Early parabiotic experiments demonstrated that *ob/ob* mice were unable to produce a satiety factor but could respond to such a factor from a donor animal (Coleman 1973; Coleman 1978). Positional cloning identified the leptin gene on chromosome 6 as the site of the ob mutation (Friedman *et al.* 1991; Zhang *et al.* 1994). This 16kDa adipokine is produced by white adipose tissue and acts on the hypothalamic ventral median nucleus to produce its prominent anorexic effects. In physiological states, leptin mediates an innate adaptive neuroendocrine response to starvation. As adipose tissue is depleted, leptin levels fall. This drives a desire to seek food and suppresses the thyroid, growth hormone, adrenal and reproductive endocrine axes (Ahima *et al.* 2000). Leptin influences numerous physiological processes and can directly affect the inflammatory response. Leptin-deficient *ob/ob* mice exist in a state of perceived starvation (Fantuzzi & Faggioni 2000). When give free access to standard chow, young *ob/ob* mice overeat, become obese and develop steatosis. This model has been extensively studied, although the ob mutation is not prevalent in the human obese, NASH population and leptin levels correlate poorly with the development of NASH (Chalasani *et al.* 2003).

Reflecting the plural activity of leptin, the *ob/ob* mouse is a complex model of obesity-related steatosis. Lipid and carbohydrate metabolism is deranged in several ways. The expanded white adipose tissue mass in *ob/ob* mice and increased expression of TNFα promote adipose tissue lipolysis releasing LCFAs. Increased circulating LCFAs are delivered to the liver (Figure 2, *1). In addition, SREBP-1c is activated and accumulates in *ob/ob* hepatocyte nuclei promoting fatty acid synthase activity and *de novo* synthesis (Figure 2, *2) (Shimomura *et al.* 1999). The increased synthesis and storage of lipid in the liver coincident with expanded adipose tissue stores contribute to hepatic steatosis and obesity.

There is conflicting evidence regarding the activity of hepatocyte fatty acid oxidation pathways in *ob/ob* mice (Figure 2, *3). Supporting the view that β-oxidation may not be appropriately upregulated in this setting, increased PPARα activity in *ob/ob* mice has not been demonstrated (Koteish & Diehl 2001). Although one proteomic study comparing *ob/ob* and lean mice demonstrates increased expression of peroxisomal 3-ketoacyl-CoA thiolase b and acyl-CoA oxidase and mitochondrial acyl-CoA dehydrogenase (Edvardsson *et al.* 2003), other studies indicate that there appears to be little increase in mitochondrial β-oxidation in *ob/ob* mice (Brix *et al.* 2002). However, investigators have demonstrated increased microsomal ω-oxidation activity (CYP2E1 and CYP4A) (Enriquez *et al.* 1999). Taken together, these findings suggest that there is little if any increase in hepatocyte β-oxidation in *ob/ob* mice, and that any increase is insufficient to dispose of the greater fatty acid load, necessitating the activation of the alternate microsomal ω-oxidation pathways.

Unlike the human NAFLD population, *ob/ob* mice do not spontaneously progress from steatosis to steatohepatitis. Ob mice require a 'second hit' to be administered to trigger progression to steatohepatitis. This may be provided by exposure to small doses of lipopolysaccharide endotoxin (LPS) that are insufficient to cause ill effects in control animals (Yang *et al.* 1997; Faggioni *et al.* 1999), ethanol exposure or hepatic ischaemia-reperfusion challenge which all provoke a severe steatohepatitis and frequently acute mortality (Chavin *et al.* 1999; Koteish & Diehl 2001). These findings underpin the assertion that steatosis sensitizes the liver to oxidative stress and free radical-mediated damage.

Leptin deficiency suppresses both the innate (monocyte/macrophage mediated) and acquired (T-lymphocyte mediated) immune responses. Hepatic depletion of natural killer T cells (NKT cells) favours a pro-inflammatory, anti-fibrotic type 1 T-helper cell (Th-1) lymphocyte polarization and so makes the steatotic liver vulnerable to LPS toxicity (Guebre-Xabier *et al.* 2000). It is widely accepted that TGFβ has an important regulatory role in the development of liver fibrosis (Friedman 2003). Defects in the production or post-translational modification of TGFβ prevent expression of TGFβ-dependent genes including pro-collagen type 1 and limit fibrogenesis. Studies demonstrate that leptin is necessary for the release of TNFα and the activation of TGFβ in response to liver injury. Hence, *ob/ob* mice are resistant to hepatic fibrosis, even when exposed to carbon tetrachloride (Honda *et al.* 2002; Leclercq *et al.* 2002; Leclercq *et al.* 2003). This protective effect may be reversed by concomitant leptin administration.

Using the *ob/ob* model, leptin treatment has also been shown to increase levels of norepinephrine which directly activates hepatic stellate cells. Similarly, treating *ob/ob* mice with norepinephrine has been shown to reset the Th-1/Th-2 balance in favour of a profibrotic Th-2 response with increased IL-4, IL-10 and TGFβ cytokine levels. Despite persistent leptin deficiency, norepinephrine treatment allows restitution of the depleted hepatic natural killer T-cell population and limits the Th-1-promoted LPS-induced toxicity while increasing collagen deposition and hepatic fibrosis (Oben *et al.* 2003; Li *et al.* 2004).

The effects of leptin deficiency on so many aspects of physiology increase the complexity of studies using this line. Similarly, the limited fibrotic capacity of a leptin-deficient model means that it is best suited to studies investigating the mechanisms behind the development of steatosis and the transition to steatohepatitis. Recent work demonstrates that the apparent flaws in this model can be turned to advantage, providing new insights into stellate cell function and the progression to fibrosis.

A model of genetically determined leptin resistance (the db/db mouse)

Mutations in the diabetes (db) gene, mapped to mouse chromosome 4, result in an autosomal recessive diabetic, obese phenotype similar to the *ob/ob* mouse (Hummel *et al.* 1966). The *db/db* mice (and the *fa/fa* rat) have normal or elevated levels of leptin but are resistant to its effects. Studies have shown that the *db* gene encodes the leptin receptor (OB-R) which is structurally similar to a class I cytokine receptor (Tartaglia *et al.* 1995). On binding leptin, the receptors form tetrameric complexes (two receptors with two molecules of leptin) and the intracellular domain undergo ligand-induced conformational change. Several alternative splice variants with a single transmembrane domain and a cytoplasmic region of variable length have been described (Fantuzzi & Faggioni 2000). The short OB-Ra isoform has not been shown to have any signalling activity. In contrast, the OB-Rb isoform has a long intracytoplasmic region that contains signal transduction motifs that activate the JAK/STAT protein kinase signal transduction cascade (Ghilardi *et al.* 1996). C57BL/Ks *db/db* mice carry a sequence insertion at the 3′ end of the mRNA transcript exactly where the OB-Ra and OB-Rb transcripts diverge. This insertion contains a stop codon that leads to the premature termination of the OB-Rb long intracellular signalling domain, loss of function and consequently leptin resistance (Chen *et al.* 1996).

Models of genetically determined increased circulating LCFAs

The models described so far demonstrate the genesis of steatosis associated with obesity, but fatty acid delivery to the liver in excess of oxidative requirements can induce steatosis, irrespective of adipose tissue mass. This has been demonstrated by modifying how fatty acids are partitioned between body tissues in non-obese animals. The transmembrane protein CD36 (fatty acid translocase) is an important fatty acid transporter expressed in peripheral tissues including muscle and adipose tissue. CD36 null mouse exhibits elevated circulating LCFA and TG levels and hepatic IR due to impaired fatty acid storage; affected mice develop steatosis and fail to suppress hepatic gluconeogenesis (Coburn *et al.* 2000; Goudriaan *et al.* 2003). In contrast, HSL-deficient animals and mice with targeted over expression of muscle lipoprotein lipase have reduced levels of circulating LCFAs and are not steatotic (Voshol *et al.* 2001; 2003).

Models of acquired increased hepatic LCFA uptake and *de novo* lipogenesis

The strong association between obesity and NAFLD has prompted study of the effects of diet on the development of steatosis and steatohepatitis in mouse models. Increased dietary supply of fat or carbohydrate to the liver may promote steatosis by increasing hepatic lipid uptake or *de novo* synthesis (Figure 2, *1 and *2). Complex traits such as obesity and fatty liver disease are influenced as much by subtle genetic variations between the strains studied (Silver 1995) as by the formula of the diet they are fed. The effects of sustained consumption of an elevated fat content diet (HFD) on inbred

mouse strains are reported elsewhere, however, much of the existing literature is primarily concerned with effects on insulin sensitivity, glucose tolerance and obesity rather than hepatic deposition (West *et al*. 1992). A comprehensive database describing the biochemical and hepatic effects of 8-week consumption of an 'atherogenic' 15% fat diet in 42 different inbred strains of mouse and 8 mutant lines may be found in the Mouse Phenome Database (http//:www.jax.org/phenome) (Grubb *et al*. 2004).

HFD fed C57BL/6 mice are considered a valuable tool for investigating the metabolic syndrome (Collins *et al*. 2004; Winzell & Ahren 2004). With ageing, C57BL/6 mice are genetically prone to obesity, hyperinsulinaemia and glucose intolerance irrespective of diet. This phenotype may be exacerbated and florid mixed micro- and macrovesicular steatosis provoked by feeding a 55% HFD for 6 months. These observations may in part be explained by a spontaneous mutation in the nicotinamide nucleotide transhydrogenase gene carried by C57BL/6 mice which induces glucose intolerance (Toye *et al*. 2005). In contrast, 129S6/SvEvTac mice develop a microsteatotic phenotype only after receiving HFD (Biddinger *et al*. 2005). Expression of SREBP-1c, SREBP-2 and Stearoyl-CoA desaturase 1 (an enzyme marker of hepatic lipid synthesis) was increased by HFD in both groups, but C57BL/6 mice was more sensitive to its effects (Biddinger *et al*. 2005). Studies using gastrostomy-based HFD overfeeding of C57BL/6 mice also demonstrate inducible steatohepatitis associated with IR, obesity, glucose intolerance and raised plasma ALT levels are associated with induction of SREBP-1c, peroxisome proliferator receptor γ (PPARγ) and Liver X receptor α (LXRα) expression and reduced expression of PPARa (Deng *et al*. 2005).

Diets with elevated carbohydrate content have also been used to provoke steatosis in mouse models. C57BL/6 mice fed a 65% sucrose diet for 8 weeks have been shown to exhibit obesity, IR and macrovesicular steatosis (Feldstein *et al*. 2003). HFD and/or high sucrose diet induced steatosis in C57BL/6 mice leads to hepatic α cell depletion, a similar Th-1 polarization to that seen in *ob/ob* mice and exaggerated LPS sensitivity (Li *et al*. 2005). This observation is significant, because it confirms that pathogenic processes underlying increased LPS sensitivity in *ob/ob* mice may be replicated in an aetiologically appropriate model to man, however, the mechanisms for the NKT cell depletion remain undefined (Jones 2005). HFD-induced steatohepatitis is also associated with increased hepatic expression of the endotoxin-induced macrophage receptor with a collagenous structure (MARCO) and portal endotoxin levels in some mouse strains suggesting that HFD may not only sensitize the liver to LPS but also increase portal delivery of LPS (Yoshimatsu *et al*. 2004).

Models of genetically determined hepatic lipogenesis

Targeted over expression of the insulin-controlled transcription factor SREBP-1 promotes hepatic lipogenesis. Two non-obese transgenic mouse models (PEPCK-nSREBP-1a mice and aP2-nSREBP-1c mice) with severe hepatic steatosis have been described (Shimano *et al*. 1996; Shimomura *et al*. 1998b). PEPCK-nSREBP-1a mice over express a truncated version of SREBP-1a in the liver under the control of a phosphoenolpyruvate carboxykinase promoter (Shimano *et al*. 1996). These animals exhibit histological steatosis but not steatohepatitis or dyslipidaemia, although ALT levels are elevated. aP2-nSREBP-1c mice over express SREBP-1c in adipose tissue. These animals have a lipoatrophic phenotype associated with steatosis (Shimomura *et al*. 1998b). Studies have shown that SREBP-1-mediated hepatic lipogenesis proceeds, even in the presence of profound IR (Shimomura *et al*. 1999).

Carbohydrate-mediated lipogenesis is transcriptionally regulated by ChREBP (Yamashita *et al*. 2001). Targeted disruption of the *ChREBP* gene in a mouse model has been shown to produce a 50% reduction in the expression of key enzymes that mediate lipogenesis (Iizuka *et al*. 2004). These data suggest that both hyperglycaemia and hyperinsulinaemia (even on a background of IR) promote hepatic steatosis. The study of rodent models has also identified that PPARγ contributes to the development of steatosis and that liver-specific deletion of PPARγ reduces steatosis.

Models of genetically determined reduced β-oxidation

Several induced and spontaneous genetic mutations that are associated with steatosis due to defective β-oxidation have been described. The mutations all affect key regulatory transcription factors, the import of LCFAs into mitochondria and enzymatic activity within the oxidative cascade. Although these models establish causality in that these mutations may provoke steatosis, few exhibit steatohepatitis or provide a stable tool for NAFLD research.

This problem with these models is exemplified by the naturally occurring juvenile visceral steatosis (JVS) mouse. In this model, systemic carnitine deficiency is induced by a mutation in the carnitine transporter gene *Octn2* and leads to failure of fatty acid transport into mitochondria for β-oxidation (Kuwajima *et al*. 1991). This model develops extreme steatosis within days of birth. Similarly, acyl-CoA oxidase ($AOX^{-/-}$) mutant mice carry a deletion in a key enzyme in peroxisomal β-oxidation. Initially, animals are phenotypically normal but over an 8-week period, mice develop severe steatosis, but this

then spontaneously resolves as steatotic hepatocytes are replaced. Mice carrying a homozygote PPARα knockout do not accumulate fat under normal fed conditions but fail to upregulate fatty acid oxidation and so develop severe steatosis when fatty acid delivery to the liver is increased by fasting (Kersten *et al*. 1999; Koteish & Diehl 2001).

A recently described model of NAFLD exhibits a strong phenotypic resemblance to the human disease. MTP is a key enzyme mediating mitochondrial β-oxidation. Homozygous carriage of an MTPα mutation causes perinatal death. Recently published data demonstrate that carriage of the heterozygous mutation is associated with a progressive, age-related elevation of serum ALT levels. By 9–10 months, mutant mice exhibit hepatic steatosis, basal hyperinsulinaemia and evidence of IR and impaired glucose tolerance during glucose tolerance testing (Ibdah *et al*. 2001; Ibdah *et al*. 2005). Mutant animals have also been shown to have higher antioxidant activity of total superoxide dismutase and lower glutathione levels as well as increased CYP2E1 expression, consistent with increased hepatic oxidative stress.

Models of acquired reduced β-oxidation (methionine/choline deficiency)

Impaired β-oxidation may be induced by drugs including the oestrogen antagonist Tamoxifen or the CPT1 inhibitor etomoxir (Koteish & Diehl 2001), however, the main research model in which an acquired defect in mitochondrial β-oxidation is used to induce steatosis is based on feeding a diet deficient in methionine and choline.

Choline is an FDA-classified essential nutrient with roles in cell membrane integrity, transmembrane signalling, phosphatidylcholine synthesis, neurotransmission and methyl metabolism. The role of dietary choline deficiency in promoting hepatic steatosis and reduced plasma VLDL levels is well established in the literature [reviewed in (Zeisel & Blusztajn 1994)]. This was thought to be due to impaired synthesis of phosphatidylcholine resulting in diminished VLDL assembly and secretion and consequently reduced TG clearance (Yao & Vance 1990) (Figure 2, *4). Recent observations now cast doubt on this explanation and suggest further mechanistic study is required to define the role of choline deficiency in steatosis (Kulinski *et al*. 2004). Mice fed a diet that is deficient in both choline and the essential amino acid methionine (MCD) develop inflammation and hepatic fibrosis in addition to simple steatosis (Weltman *et al*. 1996). The magnitude of the effect of this diet varies according to the species, strain and gender of animals studied (Kirsch *et al*. 2003). Evidence suggests that MCD impairs mitochondrial β-oxidation and leads to induction of alcohol-inducible CYP2E1 expression (Weltman *et al*. 1996): a finding later confirmed in a cohort of NASH patients (Weltman *et al*. 1998) (Figure 2, *3). These findings support the hypothesis that alcoholic and non-alcoholic steatohepatitis may share pathogenic mechanisms. ROS produced by CYP2E1 ω-oxidation, coupled with depletion of hepatic anti-oxidants (e.g. reduced glutathione and *s*-adenosylmethionine), promotes oxidative stress and induces an histological steatohepatitis. The histological features are associated with elevated plasma TNFα levels (Chawla *et al*. 1998) and may be ameliorated by treatment with pentoxifylline (Koppe *et al*. 2004). The MCD diet induces significantly greater ROS production, mitochondrial DNA damage and apoptotic cell death than many of the other mouse models of NAFLD (Gao *et al*. 2004).

The MCD model is arguably the best-established model with which to study the inflammatory and fibrotic elements of the NAFLD spectrum. Despite this, there is little evidence to support the assertion that this model replicates either the phenotype or the pathogenic mechanisms of metabolic syndrome-related NAFLD. In contrast to human fatty liver disease, animals fed the MCD diet are cachectic (50% weight loss compared with control mice by 10 weeks), have low plasma TG levels and reduced liver weight/body weight ratio. The histological distribution of hepatic steatosis differs from the pattern seen in humans where a periportal rather than perivenous deposition is seen (Koteish & Diehl 2001). MCD fed mice also exhibit a markedly more elevated plasma ALT level than that seen in patients with NASH. This model is also not overtly insulin resistant (Rinella & Green 2004), although there has been some recently published data indicating that CYP2E1 expression and consequent oxidative stress may impair insulin signalling by reducing tyrosine phosphorylation and increasing serine phosphorylation of IRS-1 (Schattenberg *et al*. 2005).

Combined models of genetically and environmentally determined NAFLD

Several groups have attempted to bridge the phenotype gap that exists between available models and the human disease. This work has involved combining genetic and dietary manipulation to produce more severe, accelerated steatohepatitis or fibrosing steatohepatitis. The literature reports a number of such combination models including *Abcb11* mutant mice fed an MCD diet (Sundaram *et al*. 2005), apolipoprotein E mutant mice fed an high fat diet (Tous *et al*. 2005) and PPARα null mice fed MCD (Kashireddy & Rao 2004). These models are all characterized by the development of histological steatohepatitis.

Feeding steatotic db/db mice, the MCD diet is shown to produce an accelerated fibrosing steatohepatitis (Sahai *et al.* 2003). In this model, hepatic pro-collagen type 1 mRNA levels were found to be 10-fold increased in *db/db* mice while non-steatotic *db/m* mice had a 4-fold rise, and *ob/ob* mice showed no increase in fibrogenesis with MCD feeding. The authors conclude that their data demonstrate the development of an obese/diabetic experimental model of progressive NASH and suggest an important role for the short-form leptin receptor in steatohepatitis.

Our own group has approached this problem in a different way. To identify relevant pathogenic genes and novel models of steatohepatitis, we have collaborated in a random N-ethyl-N-nitrosourea chemical mutagenesis screen and assayed offspring for insulin resistant, diabetic and NASH phenotypes. Initial phenotypic data from one line derived from this screen demonstrate a stable model of impaired glucose tolerance/IR with increased body weight and histological evidence of NASH strongly resembling the human NASH phenotype (Anstee *et al*. 2003).

Conclusion

The increasing prevalence of obesity, diabetes and IR and NAFLD within Western society makes research in this field imperative. It is only through better understanding of pathogenic mechanisms that novel therapies targeting both the steatotic/steatohepatitic process and fibrogenesis may be discovered. Animal models of NAFLD have provided phenotypically consistent tools with which disease pathogenesis may be dissected allowing us to appreciate NAFLD as one facet of a systemic disorder rather than an organ-specific phenomenon.

Acknowledgements

Q. M. Anstee is a Medical Research Council funded Clinical Research Fellow.

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