Model of nonalcoholic steatohepatitis1–4

Charles S Lieber, Maria A Leo, Ki M Mak, Youqing Xu, Qi Cao, Chaoling Ren, Anatoly Ponomarenko, and Leonore M DeCarli

ABSTRACT

Background: Obesity and diabetes are frequently associated with nonalcoholic steatohepatitis (NASH), but studies have been hampered by the absence of a suitable experimental model.

Objective: Our objective was to create a rat model of NASH.

Design: Sprague-Dawley rats were fed a high-fat, liquid diet (71% of energy from fat, 11% from carbohydrates, 18% from protein) or the standard Lieber-DeCarli diet (35% of energy from fat, 47% from carbohydrates, 18% from protein). The diets were given ad libitum or as two-thirds of the amount consumed ad libitum.

Results: Rats fed the high-fat diet ad libitum for 3 wk developed panlobular steatosis, whereas those fed the standard diet had few fat droplets. Accordingly, total lipid concentrations with the high-fat and standard diets were 129.9 ± 9.1 (± SEM) and 66.7 ± 4.6 mg/g liver, respectively (P < 0.001). The high-fat diet caused abnormal mitochondria and mononuclear inflammation, which were accompanied by increased hepatic tumor necrosis factor α (TNF-α; P < 0.001), TNF-α messenger RNA (mRNA) (P < 0.001), collagen type 1, and α1(I) procollagen mRNA (P < 0.001). In addition, these rats had increased cytochrome P4502E1 (CYP2E1) mRNA (P < 0.001), which was accompanied by CYP2E1 induction (P < 0.001) and oxidative stress with increased 4-hydroxynonenal (P < 0.001).

Plasma insulin was elevated, which reflected insulin resistance, a NASH pathogenic factor. Rats fed a restricted high-fat diet developed only mild steatosis with attenuated biochemical changes, whereas those given a restricted standard diet had normal livers.


KEY WORDS Model of nonalcoholic steatohepatitis, mitochondria, cytochrome P4502E1, tumor necrosis factor α, collagen, 4-hydroxynonenal, insulin resistance, high-fat diet, dietary restriction, oxidative stress

INTRODUCTION

Obesity and diabetes are common in our aging population and are frequently associated with nonalcoholic fatty liver disease, which includes nonalcoholic fatty liver and nonalcoholic steatohepatitis (NASH). Nonalcoholic fatty liver is usually considered benign, but NASH is increasingly recognized as a precursor to more severe liver disease and sometimes evolves into “cryptogenic” cirrhosis (1). In the general population, the prevalence of nonalcoholic fatty liver disease and NASH averages 20% and 2–3%, respectively (2), which makes these conditions the most common liver diseases in the United States. Fibrosis may or may not be present in persons with these diseases. No therapy for NASH has been proven to be clearly effective (3–6). Indeed, the study of the pathogenic or therapeutic factors involved in NASH has been hampered by the absence of a suitable experimental model: the models available either lack one of the pathogenic factors, such as cytochrome P4502E1 (CYP2E1) induction (7); require rats to be treated for a long time (up to 1 y) (8); use rodents with a genetic defect (9); or involve feeding rats a diet lacking choline and methionine (10), which creates a nutritional deficiency that is not common in patients with NASH but to which rodents are selectively sensitive.

The aim of the present study was to produce a practical and accurate experimental model of NASH by designing a diet that contained all essential nutrients in adequate amounts and that was palatable enough to be consumed by rats in sufficient quantities to lead to increased body weight. We hoped that this model would reproduce the key features of NASH in humans.

MATERIALS AND METHODS

Animals and diets

Male Sprague-Dawley rats, which were purchased from Charles River Laboratories (Wilmington, MA), were individually housed and fed either a standard liquid diet with 35% of energy derived from fat, 18% from protein, and 47% from carbohydrates (11) or a high-fat liquid diet (12) with 71% of energy derived from fat, 11% from carbohydrates, and 18% from protein. The diets were purchased from Dyets Inc (Bethlehem, PA). The overall compositions of the liquid diets are shown in Table 1. The standard diet has the same fat content as the average “normal” US diet (13, 14), an amount recommended as “healthy” by the Institute of Medicine (15). The rats were fed the diets either ad libitum (22 rats in each diet group) or in an amount restricted to two-thirds of the amount spontaneously consumed (at least 12 rats in each group). They were killed in the fed state either by

1 From the Section of Liver Disease and Nutrition, Bronx VA Medical Center and Mt Sinai School of Medicine, New York.
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3 Supported by NIH grant AA11115, the Department of Veterans Affairs, and the Kingsbridge Research Foundation.
4 Reprints not available. Address correspondence to CS Lieber, VA Medical Center (151-2), 130 West Kingsbridge Road, Bronx, NY 10468. E-mail: lieberci@aol.com.
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and 4° 0.025; chromium, 0.5; chloride, 390; sulfate, 250; and fluoride, 0.25 (12).

125; manganese, 13.5; iron, 8.8; copper, 1.5; zinc, 7.5; iodine, 0.05; selenium,
DNA labeling kit (Amersham, Arlington Heights, IL).

Measurement of CYP2E1, tumor necrosis factor α, collagen, and their messenger RNAs

For the measurement of CYP2E1 protein, microsomal proteins (15 μg) were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (17) and electrically transferred to nitrocellulose membranes (18). The Western blots were reacted with rabbit anti-hamster P4502E1 immunoglobulin G, which was produced in our laboratory, and immunostained by using goat anti-rabbit immunoglobulin G, conjugated to alkaline phosphatase. The blots were incubated with Immun-Star chemiluminescent substrate and enhancer (Bio-Rad Laboratories, Hercules, CA). The nitrocellulose membranes were then exposed to X-ray film and quantified by using the Evaluating Image Analysis Systems MCID (Imaging Research Inc, St Catharines, Canada). CYP2E1 was expressed in arbitrary units. Protein was measured with the BCA Protein Assay kit (Pierce, Rockford, IL).

Oxidative stress and other chemical measurements

In the liver, oxidative stress was assessed by the concentration of 4-hydroxynonenal, which was measured by using gas chromatography–mass spectrometry according to van Kuijk et al (22) as described before (23). Measurement of total lipids was carried out according to Amenta (24).

Histopathology

Formalin-fixed and paraffin-embedded livers were processed routinely for hematoxylin and eosin, chromotrope aniline blue, and Sirius red staining. Lobular inflammatory activity was determined according to Nouchi et al (25) as 1 focal collections of mononuclear inflammatory cells, 2 diffuse infiltrates of mononuclear inflammatory cells, 3 focal collections of polymorphonuclear cells in addition to mononuclear cell infiltrates, or 4 diffuse infiltrates of polymorphonuclear cells in the parenchymal area. These criteria are modifications of those used by Knodell et al (26) in patients. Tissues were also processed for electron microscopy with standard techniques as previously published (27).

Statistical analysis

Results are expressed as means ± SEMs and were analyzed by using Student’s t test (for the data in Table 2) or one-way

### TABLE 1
Composition of the liquid diets

<table>
<thead>
<tr>
<th>Component</th>
<th>Standard diet</th>
<th>High-fat diet</th>
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<tr>
<td></td>
<td>g/L</td>
<td>g/L</td>
</tr>
<tr>
<td>Casein</td>
<td>41.4</td>
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</tr>
<tr>
<td>L-Cystine</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Corn oil</td>
<td>8.5</td>
<td>48.5</td>
</tr>
<tr>
<td>Olive oil</td>
<td>28.4</td>
<td>28.4</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Dextrose maltose</td>
<td>115.2</td>
<td>25.6</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.53</td>
<td>0.53</td>
</tr>
<tr>
<td>Fiber</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Xanthan gum</td>
<td>3.0</td>
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| Composition of the liquid diets $^1$ |

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$^1$ Each diet contained 1000 kcal and the following vitamins: thiamine hydrochloride, 1.5 mg; riboflavin, 1.5 mg; pyridoxine hydrochloride, 1.75 mg; nicotinic acid, 7.5 mg; calcium pantothenate, 4.0 mg; folic acid, 0.5 mg; biotin, 50 μg; vitamin B-12, 25 μg; β-aminobenzoic acid, 12.5 mg; inositol, 25 mg; vitamin A, 6000 IU; vitamin D, 400 IU; vitamin E, 30 IU; and vitamin K, 125 μg. Each diet also contained the following minerals (in mg): calcium, 1300; phosphorus, 1000; sodium, 255; potassium, 900; magnesium, 125; manganese, 13.5; iron, 8.8; copper, 1.5; zinc, 7.5; iodine, 0.05; selenium, 0.025; chromium, 0.5; chloride, 390; sulfate, 250; and fluoride, 0.25 (12).

### TABLE 2
Energy consumption, body weight, and liver weight of rats fed the standard or the high-fat diet at libitum

<table>
<thead>
<tr>
<th></th>
<th>Standard diet group</th>
<th>High-fat diet group</th>
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<tbody>
<tr>
<td></td>
<td>(n = 22)</td>
<td>(n = 22)</td>
</tr>
<tr>
<td>Total energy kcal</td>
<td>1863 ± 70</td>
<td>1767 ± 42</td>
</tr>
<tr>
<td>kcal/d</td>
<td>91 ± 2.9</td>
<td>87 ± 1.7</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>Starting</td>
<td>134 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Final</td>
<td>311 ± 9.8</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>11.8 ± 0.51</td>
<td>12.1 ± 0.38</td>
</tr>
<tr>
<td>(g/100 g body wt)</td>
<td>3.79 ± 0.09</td>
<td>3.94 ± 0.07</td>
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$^1$ x ± SEM. There were no significant differences between the groups.

exsanguination (under light pentobarbital anesthesia) or by decapsulation after 3 wk. All animal procedures were carried out in accordance with the Institutional Guidelines.

### Organelle preparation

Livers were homogenized and centrifuged at 8700 × g and 40 °C for 10 min to obtain the mitochondrial pellet. The postmitochondrial supernatant fluid was recentrifuged at 100 000 × g and 4 °C for 1 h to separate the microsomes from the cytosol (16). Aliquots of the homogenates and the microsomes were frozen in liquid nitrogen and stored at −80 °C.

### Measurement of CYP2E1, tumor necrosis factor α, collagen, and their messenger RNAs

For the measurement of CYP2E1 protein, microsomal proteins (15 μg) were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (17) and electrically transferred to nitrocellulose membranes (18). The Western blots were reacted with rabbit anti-hamster P4502E1 immunoglobulin G, which was produced in our laboratory, and immunostained by using goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase. The blots were incubated with Immun-Star chemiluminescent substrate and enhancer (Bio-Rad Laboratories, Hercules, CA). The nitrocellulose membranes were then exposed to X-ray film and quantified by using the Evaluating Image Analysis Systems MCID (Imaging Research Inc, St Catharines, Canada). CYP2E1 was expressed in arbitrary units. Protein was measured with the BCA Protein Assay kit (Pierce, Rockford, IL).

Plasma concentrations of alanine aminotransferase and aspartate aminotransferase were measured at 30 °C by using Sigma kits (Sigma Diagnostic, St Louis), and insulin concentrations were measured by using a rat insulin radioimmunoassay kit at 4 °C (Linco Research Inc, St Charles, MO).
analysis of variance followed by the Bonferroni multiple comparisons test (for all other analyses). \( P < 0.05 \) was considered statistically significant. All statistical analyses were performed with the use of INSTAT version 3 (Graph Pad Software, San Diego).

RESULTS

Effects of standard and high-fat diets given ad libitum

There were no significant differences between the 2 diet groups in total calories consumed and starting and final body weight as analyzed by Student’s \( t \) test (Table 2). The weight gain achieved with these 2 liquid diets fed ad libitum was greater than that observed with a nonpurified stock diet (28). The rats that were fed the standard diet for 3 wk developed modest steatosis (Figure 1A), whereas steatosis was much more striking in the rats that were fed the high-fat diet (Figure 1B). This higher steatosis in the high-fat diet group was confirmed by total lipid concentrations, which were 66.7 ± 4.6 and 129.9 ± 9.1 mg/g liver in the standard and high-fat diet groups, respectively (\( P < 0.001 \)). Inflammation was also more prominent with the high-fat diet (Figure 1D) than with the standard diet (Figure 1C). This was corroborated by morphologic analysis of the abundance of inflammatory cells (2.6 ± 0.3 and 1.4 ± 0.2 arbitrary units in the high-fat and standard diet groups, respectively; \( P < 0.005 \)). The greater inflammation in the high-fat diet group was accompanied by significantly higher concentrations of hepatic TNF-\( \alpha \) (Figure 2) and its mRNA (477.9 ± 13.3 and 841.0 ± 31.2 amol/mg liver in the standard and high-fat diet groups, respectively; \( P < 0.001 \)).

In the rats fed the high-fat diet, electron microscopy showed abnormal mitochondria with degenerative changes, including rarefied matrix and loss of cristae (Figure 3A). The higher number of abnormal mitochondria in the high-fat diet group than in the standard diet group was confirmed by morphometry, which showed frequencies of abnormal mitochondria of 60.0 ± 13.8% and 15.0 ± 7.5% in the high-fat and standard diet groups, respectively (\( P < 0.05 \)). Herniated mitochondria were also observed in the rats fed the high-fat diet (data not shown).

Compared with the standard diet, the high-fat diet also resulted...
in significantly higher hepatic concentrations of CYP2E1, as measured on Western blots (1.31 ± 0.08 compared with 0.80 ± 0.07 arbitrary units; P < 0.001). However, restriction diminished the effect of the high-fat diet, and TNF-α concentrations were significantly lower in the rats fed the high-fat diet in restricted amounts than in the rats fed the same diet ad libitum (P < 0.001).

Effects of dietary restriction

As expected, compared with ad libitum feeding, dietary restriction led to lower gains in body weight: 4.5 ± 0.1 compared with 8.4 ± 1.2 g/d (P < 0.001) with the standard diet and 4.7 ± 0.1 compared with 8.2 ± 1.1 g/d (P < 0.001) with the high-fat diet. Dietary restriction also attenuated the hepatopathology (Figure 8): only modest fat accumulation and inflammation occurred with the restricted high-fat diet (Figure 8B), whereas florid lesions developed when the same diet was fed ad libitum (panels B and D in Figure 1 and insets in Figure 8). With the restricted standard diet, liver histology was normal (Figure 8A).

Consistent with the lower inflammation, TNF-α concentrations in the high-fat diet group were not as high when the diets were restricted as when they were given ad libitum (Figure 2). Dietary restriction produced a similar trend in TNF-α mRNA concentrations, which were lower when the diets were restricted than when they were given ad libitum [333.8 ± 31.0 amol/mg liver (NS) with the standard diet and
No significant differences in aspartate aminotransferase and alanine aminotransferase concentrations were observed between the ad libitum and the restricted diets (data not shown).

There were fewer abnormal mitochondria with the restricted high-fat diet (frequency of 18.0 ± 11.4%) than with the ad libitum high-fat diet (frequency of 60.0 ± 13.8%; P < 0.05). No abnormal mitochondria were seen with the restricted standard diet.

Similarly, CYP2E1 induction with the high-fat diet was lower when the diet was restricted than when it was given ad libitum (see above; 0.98 ± 0.08 compared with 1.31 ± 0.08 arbitrary units; P < 0.05), and the same effect was seen for CYP2E1 mRNA concentrations (Figure 4). As expected, the lower induction of CYP2E1 with dietary restriction was associated with lower 4-hydroxynonenal concentrations for both diets (Figure 5), but a significant difference remained between the high-fat and standard diets even when both were restricted: lower 4-hydroxynonenal concentrations were observed with the standard diet.

α1(I) Procollagen mRNA concentrations with the high-fat diet were also lower when the diet was restricted than when it was given ad libitum (Figure 6). There was a similar trend for collagen type 1 concentrations, which were lower when the diets were restricted than when they were given ad libitum [538.9 ± 58.8 compared with 620.7 ± 57.3 pg/mg protein (NS) with the standard diet and 718.8 ± 59.5 compared with 917.8 ± 53.2 pg/mg protein (NS) with the high-fat diet]. In addition, the plasma insulin concentrations of the rats fed the restricted high-fat diet were significantly lower than those of the rats fed the high-fat diet ad libitum but were comparable with those of the rats fed the standard diet ad libitum or in a restricted amount (Figure 7).

**DISCUSSION**

Progress in the understanding and treatment of NASH has been hampered by the lack of a practical experimental model that reproduces the key features of the disease. This gap has been filled in the present study. By feeding rats a high-fat liquid diet ad libitum, we reproduced the typical hepatic lesions of NASH, namely, steatosis (Figure 1B), inflammation (Figure 1D), and
early fibrosis, as indicated by a significant increase in collagen type 1 (see Results) and in α1(I) procollagen mRNA (Figure 6). The model also reproduces the most probable pathogenic factors. Indeed, although the pathogenesis of NASH has not yet been fully elucidated, a popular mechanism is the “two-hit” theory (12), in which the first hit is the accumulation of fatty acids in the liver due to several causes (such as obesity). One major promoting factor of this first hit is insulin resistance, which is present in most patients with NASH (29). The second hit is the peroxidation of these fatty acids because of the oxidative stress produced by different factors, such as CYP2E1 induction (30). CYP2E1 is the key enzyme of the microsomal ethanol oxidizing system, and the activity of CYP2E1 increases as part of the induction of the microsomal ethanol oxidizing system with chronic alcohol consumption (31). CYP2E1 has been shown to play a key role in the pathogenesis of alcoholic liver injury, including alcoholic steatohepatitis, because of the oxidative stress it generates (32, 33). Its pathogenic role in NASH has been recognized by a number of experts (1, 34–36). Indeed, CYP2E1 concentrations increase not only in experimental animals (8) but also in men (37, 38). CYP2E1 concentrations are invariably elevated in the liver of patients with NASH (37) because fatty acids (which increase in obesity) and ketones (which increase in diabetes) are also substrates for CYP2E1 (32); their excess upregulates CYP2E1. The resulting oxidative stress and liver injury are exacerbated by a diet low in carbohydrates and rich in fat, including unsaturated lipids, which promote CYP2E1 induction (39, 40). The diet of obese subjects is typically high in saturated fat, but it is of interest that hepatic fatty acid analyses in NASH patients indicate a significant accumulation not only of saturated fatty acids but also of unsaturated fatty acids in subjects with morbidity (41). In the present study, the diet rich in unsaturated fat and low in carbohydrates may have contributed to the rapid development of the pathologic changes of NASH within 3 wk. It is likely that more time may be needed for NASH to develop with diets containing less unsaturated fatty acids and more carbohydrates.

The model also reproduces mitochondrial lesions, and mitochondrial dysfunction contributes to oxidative stress (42). Note that many features are common to nonalcoholic fatty liver and NASH, but NASH alone is associated with mitochondrial structural defects (42). Oxidative stress causes various types of functional and structural damage and commonly increases TNF-α production in NASH (43). Obese patients with NASH also have enhanced expression of TNF-α mRNA, whereas obese patients without NASH do not (44). TNF-α increases in our model as well (Figure 2), and this proinflammatory cytokine probably contributes to the histologic inflammation (Figure 1D) and the steatosis, as shown in alcoholic steatohepatitis (45). TNF-α concentrations increased less after the restricted than after the ad libitum high-fat diet, probably because this increase in TNF-α is part of the inflammatory response, which decreases strikingly after dietary fat restriction, as illustrated in Figure 8.

In our model there was no significant increase in alanine aminotransferase concentrations, a finding that is also not uncommon in the human disease, for which a significant increase in alanine aminotransferase concentrations has been reported in less than one-third of patients (46). As with human NASH, histologic inflammation was generally present in our rat experimental model. Moreover, it was recently reported that a low normal alanine aminotransferase value does not guarantee freedom from underlying steatohepatitis, even when there is advanced fibrosis (47). In any event, taken together, the present data show that the 2 hits postulated for the pathogenesis of NASH (ie, significant hepatic steatosis and oxidative stress), as well as insulin resistance, were reproduced in the experimental model of NASH described here.

The presence and magnitude of the oxidative stress was also corroborated by increased lipid peroxidation, as shown by the appearance of corresponding breakdown products such as 4-hydroxynonenal. Hydroxynonenal is known to stimulate collagen production (48), as also reflected in the present study (Figure 5).

The availability of this new experimental model should facilitate further elucidation of the pathogenesis of NASH and the establishment of effective treatment of NASH. One such application is illustrated by the results obtained with dietary restriction, which has been shown previously to promote health, such as by delaying aging (49). The present study shows that most key features of the NASH produced by the high-fat diet were significantly attenuated by dietary restriction but that the pathology was not fully abolished (Figure 8B). By contrast, feeding the same restricted amount of the standard diet fully normalized the morphology of the liver (Figure 8A) and much of its biochemistry. Thus, future studies using the liquid diet model of NASH...
described here will not only be of interest for further pathogenic studies of this disorder, but they may also shed some light on desirable dietary therapies. Indeed, dietary approaches to treat and prevent NASH and obesity are still the subject of debate. The prevailing view that a low-fat, high-carbohydrate diet is the most appropriate diet is now being challenged by the rising popularity of a high-fat, high-protein, and low-carbohydrate (Atkins-type) diet, and it is presently not clear which diet is preferable. Recent reports showed that an Atkins-type diet had some success in relatively short-term weight-loss studies (50). However, concern remains about the long-term effects, not only in terms of sustained body weight, but also regarding negative cardiovascular side effects of a high-fat diet. Such side effects were not observed in another relatively short-term study (51), but the question whether a long-term high-fat diet leads to adverse cardiovascular effects remains. In addition, the present study also showed that possible negative effects of a high-fat diet on the liver should not be ignored. Obviously, long-term clinical trials are now needed, and it might be desirable that they be preceded by experimental studies. To that effect, variants of our present model may be useful. Indeed, our totally synthetic liquid diet provides the versatility needed to establish an optimal mix of fat, protein, and carbohydrates for achieving the desired goal while avoiding unwanted hepatic and other side effects. In any event, the present study not only shows that a high-fat diet with 71% of energy derived from fat is more deleterious to the liver than is a normal diet (with 35% of energy from fat), but it also illustrates that even when restricted, the high-fat diet produces some undesirable liver changes that are not present with equivalent amounts of the standard diet.

The key features of human NASH were successfully reproduced in rats by feeding them a high-fat, low-carbohydrate Lieber-DeCarli liquid diet ad libitum for 3 wk. This high-fat diet was more deleterious to the liver than was a calorically equivalent diet with normal fat and carbohydrate contents. Severe hepatic changes were prevented with both of the diets when their intake was restricted, but with the high-fat diet, some pathology nevertheless developed, albeit less than that observed when the diet was fed ad libitum. By contrast, the livers were normal when the standard diet was restricted. The techniques described here provide a useful tool for further studies to develop the best diet for preventing NASH.

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REFERENCES