Experimental nonalcoholic steatohepatitis compromises ureagenesis, an essential hepatic metabolic function

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Thomsen KL, Grønbæk H, Glavind E, Hebbard L, Jessen N, Clouston A, George J, Vilstrup H. Experimental nonalcoholic steatohepatitis compromises ureagenesis, an essential hepatic metabolic function. Am J Physiol Gastrointest Liver Physiol 307: G295–G301, 2014. First published June 12, 2014; doi:10.1152/ajpgi.00036.2014.—Nonalcoholic steatohepatitis (NASH) is increasing in prevalence, yet its consequences for liver function are unknown. It has been demonstrated that NASH patients tend to have a reduced activity of liver-derived coagulation factors and lower levels of albumin than patients with pure steatosis (7). Breath tests as indirect measures of liver function suggest some degree of relation between functional impairment and the extent of fatty liver lesions (8, 42). The metabolic liver function assessed more directly by the galactose elimination capacity was found to be decreased in steatosis patients (19). However, no study to date has quantified the functional metabolic liver mass in NASH by directly measuring the capacity of a process that occurs exclusively in the liver and without which health and life cannot be sustained, i.e., a so-called essential liver function.

Ureagenesis is a classic example of such an essential liver function. Importantly, it has the methodological advantage that it can be quantified, since the end product urea cannot be reutilized by mammalian enzymes. The mitochondrial-cytosolic urea cycle is the only on-demand way for the body to irreversibly dispose of nitrogen. The process, as expected, is tightly regulated to accommodate the requirements for nitrogen elimination and to secure appropriate nitrogen balance (35, 46). The impairment of ureagenesis and its normal regulation by liver disease is probably an important mechanism for several complications of liver disease (2, 25, 45). For example, in cirrhosis, the decreased capacity for ureagenesis ultimately favors hyperammonemia and hepatic encephalopathy, and the loss of normal downregulation of ureagenesis by carbohydrates entails an increased need for dietary proteins (12). It is unknown, however, whether the capacity for ureagenesis is affected by the presence of NASH.

To study the regulation of ureagenesis in vivo, it is necessary to standardize the process rate for the strong and immediate effects of substrate blood amino acid concentration. In rats, we use alanine infusion to obtain substrate-saturation, which allows for measurement of the maximum rate, i.e., the capacity of urea-nitrogen synthesis (CUNS) (14). Any change in this capacity, then, reflects nonsubstrate regulation of urea synthesis, which is exerted by changes in gene activity, liver mass, or metabolism; nonalcoholic fatty liver disease; rats; urea synthesis

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NONALCOHOLIC FATTY LIVER DISEASE (NAFLD), characterized by the excess accumulation of fat in hepatocytes, affects up to 30% of the general population. In susceptible individuals, the disease can progress from relatively bland hepatic steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis (34), with increased morbidity and mortality, beyond that attributable to the associated metabolic syndrome (16). This risk depends on the presence of NASH, but the mechanism(s) remains obscure. Analogous to the situation in cirrhosis (22, 43), however, it is a reasonable contention that the problem may be related to functional deficits in NASH-afflicted livers. In this regard, the effects of NASH per se on the metabolic function of the liver are unknown. It has been demonstrated that NASH patients tend to have a reduced activity of liver-derived coagulation factors and lower levels of albumin than patients with pure steatosis (7). Breath tests as indirect measures of liver function suggest some degree of relation between functional impairment and the extent of fatty liver lesions (8, 42). The metabolic liver function assessed more directly by the galactose elimination capacity was found to be decreased in steatosis patients (19). However, no study to date has quantified the functional metabolic liver mass in NASH by directly measuring the capacity of a process that occurs exclusively in the liver and without which health and life cannot be sustained, i.e., a so-called essential liver function.

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This study aimed to clarify the regulation of nitrogen elimination via urea in an animal model of NASH using methods describing the events from gene expression to physiological function. Early and advanced NASH was induced in rats by feeding them a Western-style high-fat high-cholesterol (HFC) diet for 4 and 16 wk, respectively.
MATERIALS AND METHODS

Animals. Female Wistar rats (body wt 220–250 g; Taconic M&B, Ejby, Denmark) were housed at 21 ± 2°C with a 12-h artificial light cycle. Three animals were housed in each cage with free access to tap water. All animals were allowed to acclimate on a standard diet for a week followed by randomization. One-half of the rats was fed a standard diet (D09052201) and the other one-half a HFC diet (D09052204) ad libitum (both from Research Diets, New Brunswick, NJ). The standard diet was composed of the following energy sources: 67 g carbohydrates (70 kcal/100 kcal), 4 g fat (10 kcal/100 kcal), and 19 g protein (20 kcal/100 kcal) per 100 g diet. The HFC diet contained 19 g carbohydrates (15 kcal/100 kcal), 39 g fat (65 kcal/100 kcal), 2 g cholesterol, and 27 g protein (20 kcal/100 kcal) per 100 g diet. The study was performed in accordance with local and national guidelines for animal welfare and approved by the institutional Animal Ethics Committee.

Design. The study consisted of two substudies conducted 4 and 16 wk after start of the diet. Seventy animals were divided into four groups. After 4 and 16 wk, respectively, we studied one group receiving the standard diet and the other receiving the HFC diet. After an overnight 12-h fast, the animals were anesthetized with a subcutaneous injection of fentanyl/fluanisone (Hypnorm; Jansen Pharma, Birkerød, Denmark) at 0.5 ml/kg and midazolam (Dormicum; La Roche, Basel, Switzerland) at 2.5 mg/kg. In 8–11 animals from each group, the blood α-amino nitrogen concentration and the blood urea concentration were measured, and CUNS was determined (cf. below). The remaining eight animals of each group were exsanguinated for blood analyses.

The liver and spleen were weighed, and slices of liver tissue from the left lobe were secured and fixed overnight in 10% buffered formalin and the next day embedded in paraffin and stained with hematoxylin-eosin and Sirius Red for histological examination. Additionally, 200 mg of liver tissue were snap-frozen in liquid N₂ and stored at −80°C. The following variables were determined: protein expression and mRNA levels of the urea cycle enzymes carbamoyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), and argininosuccinate synthetase (ASS) in liver tissue; mRNA levels of the urea cycle enzymes argininosuccinate lyase (ASL) and arginase (ARG) in liver; plasma concentrations of glucagon; serum concentrations of corticosterone, total insulin-like growth factor-I (IGF-I), and insulin; plasma concentrations of bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and serum concentrations of albumin.

Histology. All biopsy specimens were reviewed by an experienced liver histopathologist (Clouston) blinded to the clinical and laboratory data. Histological parameters were graded and staged according to standard criteria (17). Macroscopically, the livers from the rats on the HFC diet were enlarged and pale yellow. Microscopically, the livers showed severe steatosis and typical inflammatory changes of NASH at the early and late time points. Mild fibrosis was already evident in six of eight animals from the group at 4 wk (early NASH), and the stage of fibrosis increased in severity to 16 wk (advanced NASH). No morphological abnormalities were observed in the liver tissues of the control animals (Fig. 1).

CUNS. Following anesthesia, a catheter (BD Neofilon 0.7 mm; Becton Dickinson, Albertslund, Denmark) was inserted in the femoral vein for infusion of alanine. To avoid uncertainty due to urine collection, retroperitoneal nephrectomy was performed immediately before CUNS determination. This does not acutely influence the rate of urea synthesis (14). During anesthesia repeated blood samples were drawn from the retrobulbar venous plexus using heparinized micropettes (Vitrex; Hounisen Laboratory Equipment, Aarhus, Denmark). Alanine was administered according to body weight as a bolus of 0.7–1.3 ml of an 1,124 mmol/l sterile water solution followed by a constant infusion of 3.3–4.2 ml/h of a 224 mmol/l solution for 60 min by means of an injectomat (Graseby 3500; Graseby Medical, Hertfordshire, UK). Steady-state blood amino-nitrogen concentration was defined as fluctuations below 10% during at least 50 min of the study. The alanine infusion was aimed at obtaining a steady-state total blood amino-nitrogen concentration between 7.3 and 11.6 mmol/l, in which interval urea synthesis approximates its maximum in rats, i.e., it is saturated and thus independent of substrate concentration (14). After a 20-min equilibration period, blood was sampled (100 μl) for 40 min at 10-min intervals for blood urea and total amino-nitrogen determination. Blood urea was measured using the urease-Berthelot method (5), whereas the total blood α-amino-nitrogen level was measured via the dinitrofluorobenzene method (9). CUNS was calculated as the body water accumulation rate of urea corrected for intestinal hydrolysis, as previously described (14).

Liver tissue analyses. Aliquots of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the Bio-Rad Criterion Gel System (Bio-Rad, Hercules, CA). The proteins were then transferred to polyvinylidene difluoride membranes, which were then blocked with 2% nonfat dried milk (Sigma-Aldrich, Brøndby, Denmark) in Tris-buffered saline-Tween 20 (10 mM Tris, 150 mM NaCl, pH 7.8, and 0.1% Tween 20). The blocked membranes were incubated with antibodies against CPS 1, OTC, and ASS 1 (Abcam, Cambridge, UK). The membranes were washed and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Amersham, Pharmacia Biotech, Piscataway, NJ) as a secondary antibody, and the proteins were visualized by BioWest enhanced chemiluminescence (UVP, Upland, CA) and quantified using a UVP BiolImaging System (UVP). mRNA levels of urea cycle enzymes were determined by slot-blot hybridization as previously described (26, 27). Results from NASH animals are presented as relative levels compared with that in control rats.

Blood analyses. Plasma glucagon was measured by wick chromatography (28). Serum corticosterone concentrations were assessed using a specific rat RIA kit (Amersham Biosciences, Buckinghamshire, UK). Total serum IGF-I was measured after acid-ethanol extraction by an IGF-I radioimmunoassay (11). The serum insulin level was measured using an ultrasensitive rat insulin ELISA (DRG Diagnostics, Marburg, Germany), and the serum glucose level was determined by a routine analytical method. The HOMA index was calculated in accordance with a previously described model (21). Plasma bilirubin, ALT, and AST were determined by routine analytical methods. Serum albumin was evaluated using a specific rat ELISA (Alpha Diagnostic, San Antonio, TX). All of the assays that were used in this study have been validated for use in rats.

Statistical methods. Each substudy was conducted as an independent experiment with its own control group. Within each substudy, the data were analyzed using one- or two-way unpaired Student’s t-test as appropriate. The assumption of normality was checked by quantile-quantile plots. Corticosterone, total IGF-I, insulin, glucose, the HOMA index, bilirubin, ALT, and AST exhibited skewed distributions with variance heterogeneity. These data were therefore analyzed using the nonparametric Mann-Whitney Rank Sum Test. Data are presented as means ± SE. Differences were considered significant when P values were less than 0.05.

RESULTS

Animal characteristics. The liver weight increased twofold in the early NASH animals at 4 wk and more than threefold in the animals with advanced NASH at 16 wk (P < 0.001, both). The spleen weight increased by 20% in early NASH (P = 0.01) and by 40% at 16 wk (P < 0.01). The plasma ALT and AST levels were increased in early NASH (P < 0.01, both) and only AST by 16 wk (P = 0.03). Plasma bilirubin was normal in early NASH and increased fourfold in animals with advanced NASH (P < 0.001). Serum albumin was reduced in both groups (P < 0.01, both) (Table 1).
**Ureagenesis in early NASH.** The gene expression of the five urea cycle enzymes decreased by 30–48% ($P < 0.05$) (Fig. 2). The protein expression of OTC decreased markedly and selectively to 10 ± 2% ($P < 0.001$), and there was no decrease in the CPS and ASS liver proteins (Fig. 3). The whole body CUNS did not change (Fig. 4).

**Ureagenesis in advanced NASH.** Again, the gene expressions decreased, those for CPS and ARG significantly so and by 27–30% ($P = 0.02$) (Fig. 2). The protein expression of OTC was even lower at 6 ± 2% and that of CPS decreased to 63 ± 4% ($P < 0.001$). The protein expression of ASS did not change (Fig. 3). The whole body CUNS decreased by 20% [4.58 ± 0.30 vs. 5.65 ± 0.23 μmol × (min × 100 g)$^{-1}$; $P = 0.01$] (Fig. 4).

**Plasma glucagon, corticosterone, total IGF-I, insulin, glucose, and the HOMA index.** Glucagon was unaffected by NASH, whereas corticosterone was slightly decreased in early NASH ($P = 0.01$). Total IGF-I increased in advanced NASH ($P < 0.001$). Glucose, insulin, and HOMA index were unchanged by the experimental diets (Table 2).

**DISCUSSION**

The central finding of this study was that experimental NASH causes a progressive impairment of the machinery of ureagenesis, initially involving the expression of urea cycle enzymes and subsequently suppressing the whole body CUNS. Such functionally detrimental effects of NASH have not earlier been described and may have considerable pathophysiological
Our results suggest that, as steatohepatitis progresses, the ability of the liver to adjust to changing demands for nitrogen excretion and to remove potentially neurotoxic nitrogenous substances by the synthesis of urea is impaired. It is known that hyperammonemia may be present in patients with steatohepatitis (6); this finding, so far unexplained, might be related to downregulated urea synthesis, but this has not been specifically studied in humans.

There are several models of steatosis and steatohepatitis. We used a simple Western-style HFC diet rat model that displays a liver phenotype strikingly similar to human NASH (48). The diet’s 2% of cholesterol, while quantitatively unnatural in a human dietary setting, is essential for rapidly establishing our model, and there are indeed indications for an important role of cholesterol also for human NASH (20, 32). On the other hand, we have found no evidence for a direct toxic effect of cholesterol that could account for our functional findings. The livers of all the HFC-fed animals exhibited extensive structural changes mimicking the progressive phases of steatohepatitis within a few weeks. The liver weight increased probably because of marked fatty infiltration, as did the spleen weight probably from portal hypertension. Liver damage was biochemically evident by the increase in aminotransferases and by bilirubin concentrations.

We used four methods to study the regulatory steps at different levels of urea synthesis: gene expression (mRNA), protein expression, and the capacity of urea-nitrogen synthesis (CUNS). Results from NASH animals are presented as relative levels compared with that in control rats. Bars represent the means and SE. *P < 0.05 compared with controls.

Table 1. Liver and spleen weight and plasma concentrations of bilirubin, ALT, AST, and albumin in controls and in NASH animals

<table>
<thead>
<tr>
<th></th>
<th>4 wk</th>
<th>16 wk</th>
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<tr>
<td></td>
<td>Controls</td>
<td>Early NASH</td>
</tr>
<tr>
<td>Liver wt, g</td>
<td>7.6 ± 0.3</td>
<td>14.9 ± 0.8*</td>
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<tr>
<td>Liver/body wt, %</td>
<td>2.7 ± 0.0</td>
<td>5.8 ± 0.2*</td>
</tr>
<tr>
<td>Spleen wt, mg</td>
<td>930 ± 38</td>
<td>1,125 ± 53*</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>2.1 ± 0.1</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>29 ± 1</td>
<td>141 ± 37*</td>
</tr>
<tr>
<td>AST, U/l</td>
<td>9 ± 1</td>
<td>51 ± 11*</td>
</tr>
<tr>
<td>Albumin, mg/ml</td>
<td>63 ± 2</td>
<td>52 ± 2*</td>
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Data are shown as means ± SE. NASH, nonalcoholic steatohepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase. *P < 0.05 compared with controls. #P < 0.05 compared with 4 wk.
deficiencies, including OTC (37, 47), and it has been proposed that defective transport of the enzyme precursor causes the deficiency (23). Thus, despite the fact that our NASH model showed predominantly large and small droplet macronuclear fatty liver diseases, it may share a mechanism for the urea cycle suppression with the macronuclear fatty liver diseases.

Other diseases that may present with mitochondrial dysfunction and microvesicular hepatocyte infiltration are the inborn errors of urea synthesis, with OTC deficiency being the most common one. In such patients, stressful situations and large protein meals result in hyperammonemia because the excess waste of nitrogen overwhelms the ability of the deficient urea synthesis to remove the ammonia load. However, during stable periods, the activity of the enzyme, depending on the phenotype of the disease, is sufficient for a urea synthesis that allows ammonia levels within normal values. Data from the OTC-deficient “sparse fur” (spf) mice (4, 44) show that as little as 3% of normal enzyme activity is sufficient to ameliorate the severe phenotype in neonatal spf mice (3). This seems to indicate that OTC is expressed in abundance and to such an extent that the 10% remnant protein expression in our model of early NASH sufficed for a normal whole body CUNS, whereas the slightly more pronounced OTC protein reduction to only 6% in advanced NASH seemed to be functionally subthreshold and decreased CUNS by 20%. As we were not able to measure ammonia because of hyperlipidemia and did not perform behavioral studies, we do not know if this resulted in the animals becoming hyperammonemic.

The massive reduction in OTC protein expression could be a combination of reduced protein synthesis, as indicated by the reduced mRNA expression, and increased breakdown of OTC. Studies of livers of young and aging rats have shown an age-related modification of OTC resulting from posttranslational glycation (1). Formation of advanced glycation end products is increased in rat livers when exposed to a high-fat diet (18), and this could mediate an increased breakdown of OTC.

In addition to the compromised ureagenesis, there were other indications of reduced functional liver mass. The advanced NASH animals were jaundiced, indicating impairment of the liver’s excretory function. The reduced albumin may reflect low-grade inflammation with albumin as a negative acute-phase protein or decreased hepatic protein synthetic capacity. These findings are consistent with the few existing data on liver function in human NAFLD (8, 19, 42).

A possible mechanism for the genetic downregulation of urea synthesis in our NASH model is that it is secondary to increases in proinflammatory cytokines such as tumor necrosis factor-α and interleukin-6 in the liver (38). We have previously shown that these interleukins reduce the expression of all urea cycle mRNAs (40, 41). This would not, however, explain our findings, since the interleukins do not decrease the enzyme proteins or CUNS (40, 41). Serum corticosterone was slightly decreased by steatohepatitis in our rats. Corticosteroids up-regulate CUNS, so the decrease might downregulate the CUNS (36). The advanced NASH animals had increased IGF-I, and we have shown that IGF-I downregulates CUNS via decreased urea cycle mRNAs (10). Such humoral effectors may contribute toward the decreased CUNS in the present study, but, in light of the marked OTC deficiency, severe mitochondrial dysfunction seems to be the dominant cause.

| Table 2. Plasma glucagon and serum corticosterone, total IGF-I, insulin, and glucose and the HOMA index in controls and in NASH animals |
|-----------------|-----------------|-----------------|
|                 | Controls        | Early NASH      | Controls        | Advanced NASH |
| Glucagon, ng/l  | 285 ± 25        | 334 ± 55        | 218 ± 57        | 293 ± 40      |
| Corticosterone, µg/l | 760 ± 101       | 437 ± 55*       | 570 ± 88        | 343 ± 36      |
| IGF-I, µg/l     | 875 ± 23        | 827 ± 124       | 611 ± 20*       | 1,130 ± 83*   |
| Insulin, µg/l   | 0.21 ± 0.03     | 0.28 ± 0.08     | 0.37 ± 0.09     | 0.27 ± 0.10   |
| Glucose, mmol/l | 7.2 ± 0.6       | 7.8 ± 0.4       | 8.7 ± 0.8       | 6.2 ± 0.3*    |
| HOMA index      | 2.0 ± 0.4       | 2.9 ± 1.0       | 4.8 ± 1.4#      | 2.2 ± 0.8     |

Data are shown as means ± SE. IGF-I, insulin-like growth factor-I. *P < 0.05 compared with controls. #P < 0.05 compared with 4 wk.

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In conclusion, experimental NASH is not an innocuous disease. Early NASH compromises the genes and enzyme proteins involved in ureagenesis, and this is aggravated in more advanced disease. The pattern of abnormalities suggests a predominant mitochondrial involvement. The consequence is a functional reduction in the capacity for ureagenesis that impairs the ability of the body to adjust to changes in the requirements for nitrogen homeostasis. The mechanistic basis of this steatosis-dependent hepatocyte dysfunction has not yet been elucidated, as has not the possible clinical implications.

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REFERENCES


