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Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease

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Nonalcoholic fatty liver disease (NAFLD) is characterized by the accumulation of excess liver triacylglycerol (TAG), inflammation, and liver damage. The goal of the present study was to directly quantify the biological sources of hepatic and plasma lipoprotein TAG in NAFLD. Patients (5 male and 4 female; 44 ± 10 years of age) scheduled for a medically indicated liver biopsy were infused with and orally fed stable isotopes for 4 days to label and track serum nonesterified fatty acids (NEFAs), dietary fatty acids, and those derived from the de novo lipogenesis (DNL) pathway, present in liver tissue and lipoprotein TAG. Hepatic and lipoprotein TAG fatty acids were analyzed by gas chromatography/mass spectrometry. NAFLD patients were obese, with fasting hypertriglyceridemia and hyperinsulinemia. Of the TAG accounted for in liver, 59.0% ± 9.9% of TAG arose from NEFAs; 26.1% ± 6.7%, from DNL; and 14.9% ± 7.0%, from the diet. The pattern of labeling in VLDL was similar to that in liver, and throughout the 4 days of labeling, the liver demonstrated reciprocal use of adipose and dietary fatty acids. DNL was elevated in the fasting state and demonstrated no diurnal variation. These quantitative metabolic data documented that both elevated peripheral fatty acids and DNL contribute to the accumulation of hepatic and lipoprotein fat in NAFLD.

Introduction
Nonalcoholic fatty liver disease (NAFLD) is a term used to describe a spectrum of conditions characterized mainly by the histological finding of macrovesicular hepatic steatosis in individuals who consume little or no alcohol (1). The classic NAFLD patient has persistently elevated liver enzymes (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]); has negative test results for viral, congenital, and autoimmune liver diseases; and denies excessive alcohol consumption (2). Previously, fatty liver was thought to be benign. However, it has recently become clear that fatty liver is a precursor of the more advanced liver disease nonalcoholic steatohepatitis, a condition that may progress to cirrhosis in up to 25% of patients (3, 4). Fatty liver is now considered to be the hepatic component of the metabolic syndrome (5–8). The specific origin of the lipids that accumulate in the livers of patients with NAFLD will be unknown, and identifying the origin of the accumulated lipids in the livers of patients with NAFLD will be important in the prevention and reversal of this condition.

The hallmark feature of the pathogenesis of NAFLD, both histologically and metabolically, is the accumulation of triacylglycerol (TAG) in the liver. The plasma nonesterified fatty acid (NEFA) pool contributes the majority of the fatty acids that flow to the liver in the fasted state and thus provides the bulk of the fatty acids secreted by the liver in VLDL particles (9). Insulin resistance has been shown to be associated with dysregulation of adipose-derived fatty acid flux in the fasting state (10–12), and strong evidence exists demonstrating that in NAFLD patients, insulin does not suppress adipose tissue lipolysis to the same extent that it does in healthy individuals (13).

Yet other sources of fatty acids can contribute to fatty liver, and an understanding of fatty acid regulation in the fasted and fed states should take these sources into account. The potential sources of fats contributing to fatty liver are shown in Figure 1 and include peripheral fats stored in adipose tissue that flow to the liver by way of the plasma NEFA pool (pathway 1); fatty acids newly made within the liver through de novo lipogenesis (DNL) (pathway 2); and dietary fatty acids, which can enter the liver by two means: through spillover into the plasma NEFA pool (14) (pathway 3) and through the uptake of intestinally derived chylomicron remnants (15) (pathway 4). Isotope methodology has been used to quantify fatty acid (9) and TAG (9, 16, 17) flux in the fasting state in healthy, obese, and hypertriglyceridemic patients, and in one study of NAFLD (18). Using the multiple-stable-isotope approach, we have recently developed a new method to determine the fate of dietary fatty acids during the postprandial state (19, 20), and in the present work we have combined these techniques with liver biopsy to simultaneously measure all four pathways of fatty acid delivery to the liver in patients suspected of having NAFLD. These quantitative metabolic data demonstrated that both elevated peripheral fatty acid flux and DNL contribute to hepatic and lipoprotein fat in NAFLD.
Results
Clinical and laboratory values of the subjects (5 male and 4 female; 44 ± 10 years of age) revealed characteristics common to NAFLD patients (Table 1). As determined by computed tomography scans, both visceral and subcutaneous fat masses were elevated above the normal values reported by Dixon (21). Serum ALT was elevated in all subjects, serum AST was elevated in most subjects, and in most subjects, serum alkaline phosphatase was within the normal range (data not shown). In addition, fasting serum TAG concentrations revealed hypertriglyceridemia (3.2 ± 2.2 mmol/l) and the homeostasis model assessment of insulin resistance (HOMAIR), a quantitative index of insulin resistance (22), was also elevated (4.1 ± 2.8; range, 1.2–8.9). The first goal of the present study was to assess the hepatic response during transitions from fasting to feeding with respect to how fatty acids from difference sources were used for lipoprotein TAG synthesis. In the fasting state on days 2, 3, and 4, the isolation of large buoyant VLDL (Svedberg flotation unit [Sf], 60–400) revealed an average fasting concentration of 1.0 ± 0.9 mmol/l. The rate of appearance of NEFA (RaNEFA) on day 2 was 6.3 ± 2.9 μmol/kg/min. The remaining data presented at the bottom of Table 1 provide the metabolic context in which lipoprotein TAG metabolism was determined and demonstrate the expected differences between fasting and feeding (e.g., higher insulin concentrations in the fed state, lower NEFA, etc.). Changes in fatty acid sources in blood lipids are shown in Figure 2. In both states, the majority of serum NEFA originated in adipose tissue (Figure 2A). After 4 days of labeling, the average contributions of adipose fatty acids to the serum NEFA pool were 81.7% ± 8.2% and 61.7% ± 11.9% for fasted and fed states, respectively (mean ± SD; P = 0.0003). The spillover of dietary TAG into the NEFA pool was evident on the very first afternoon after subjects were fed the labeled TAG at lunch and contributed 16.3% ± 8.2% of these fatty acids. The fed-state contribution of dietary lipid to serum NEFA rose to 26.2% ± 8.9% after 4 days of labeling, a value that was significantly higher than the fasting contribution on day 5 (10.4% ± 4.9%; P = 0.0006). For newly made fatty acids, those in the serum NEFA pool could have originated from DNL in adipose or liver. The contribution of DNL to serum NEFA was lower in the fasting state than in the fed state (7.0% ± 4.0% versus 9.4% ± 4.5%; P = 0.01). The sources of fatty acids in total TAG-rich lipoproteins (tTRLS) were analyzed for comparison, and the pattern of labeling in lipoprotein TAG mirrored that of the serum NEFA pool (Figure 2B). After 4 days of labeling, the contribution of adipose- and diet-derived fatty acids to tTRL TAG synthesis changed significantly from fasting to feeding, with adipose-derived fatty acids being higher in the fasting state (60.4% ± 18.2% and 27.9% ± 10.2%, respectively; P = 0.0001) and diet-derived fatty acids being higher in the fed state (12.1% ± 4.1% versus 19.1% ± 8.3%; P = 0.01). In contrast, the contribution of DNL to tTRL TAG did not differ between fasting and feeding (22.2% ± 8.1% versus 20.4% ± 5.2%; P = 0.29). At every time point, the serum NEFA pool contributed the most to tTRL TAG, regardless of metabolic state (P = 0.0001). Evidence of recycling of the isotopes was suggested by the slow rise in sources apparent over the 4 days. The contributions of NEFA and newly made fatty acids to tTRL TAG increased with time (P = 0.007), while the contribution of dietary fatty acids to tTRL TAG showed a trend toward increasing with time (P = 0.06).

Changes in the sources of tTRL TAG immediately after consumption of a meal. Given previous observations of higher fed-state DNL (23) and the known stimulatory effect of insulin on fatty acid synthesis (24), we were surprised that fed-state lipogenesis assessed at 1500 in tTRL TAG did not differ from values obtained in the fasting state.
state. To determine if fed-state data collected at 1500 each day were truly reflective of metabolism after a meal, subjects underwent a meal test (breakfast meal). As shown in Figure 2C, these data demonstrate that the contribution of NEFA to tTRL TAG decreased in the fed state (Figure 3A) may have been due to a greater use of unlabeled fatty acids for VLDL TAG synthesis, since dietary sources (and hence chylomicrons in this fraction) would have been labeled. This observation is consistent with a greater appearance of unlabeled fatty acids apparent in VLDL TAG found in the early postprandial period in our studies of healthy subjects (M.T. Timlin and E.J. Parks, unpublished observations).

On the fifth day of the study, each subject underwent a liver biopsy, and the histological results of this procedure are presented in Table 2 (also included for comparison are subject ages and BMI). The TAG content of liver tissues ranged from not detectable by gas chromatography (below 1 μmol fatty acid) to 0.49 g per gram of tissue, which was higher than the 0.01 g per gram of tissue typically observed in healthy populations (25, 26). Histological evaluation of liver biopsies indicated steatosis in all subjects, inflammation in all but 2 subjects, and progression to fibrosis in 4 subjects (Table 2). In the liver, labeling with stable isotopes over 5 days allowed for the identification of 20.4–63.7% of hepatic TAG-palmitate. No physical or biochemical characteristics correlated with the percentage of liver TAG labeled over this period. There was, however, a strong correlation between the percentage of TAG fatty acid sources accounted for in liver and those accounted for in VLDL (Figure 3B), and a weak association between the TAG content of liver and the percentage of liver TAG accounted for by the labeling protocol ($r^2 = 0.46; P = 0.15$). The fractional synthesis rate of the total liver TAG pool ranged from 5.1% to 15.5% per day and thus the average time estimated for the near-complete turnover of this pool was 38.4 ± 15.6 days (Table 2). Although not significant, the turnover time tended to be associated with the liver TAG content ($r^2 = 0.37; P = 0.11$). To compare sources of liver TAG and TAG secreted in VLDL, we normalized the sources contributing to both TAG pools to 100% (Figure 3A). In VLDL and liver TAG, no differences between the proportions of the three fatty acid sources were found. Fatty acids originating in the NEFA pool contributed 62.4% ± 11.7% and 59.0% ± 9.9%, respectively, with newly made fatty acids contributing 22.9% ± 6.2% and 26.1% ± 6.7%, respectively, and dietary fats contributing 14.7% ± 8.5% and 14.9% ± 7.0%, respectively. Although NEFA represented the major source of liver TAG, the normalized values ranged from 45.1% to 74.3%. The variability in the use of newly made fatty acids was 12.7% to 37.0%, with the dietary contribution ranging from 4.3% to 28.0%.

### Discussion

The accumulation of fat, mainly TAG, within the hepatocyte is a prerequisite for the development of NAFLD. However, the primary metabolic abnormalities leading to lipid accretion are not well understood. Various strategies to treat NAFLD are currently focusing on improving glucose tolerance and body weight status (27–31); however, an understanding of the origins of the accumulated TAG in the liver will strengthen current treatment regimens and aid in the identification of new targets for therapy. Using a multiple-stable-isotope approach in patients who underwent a liver biopsy for diagnosis of their condition, we have documented here the sources of hepatic and lipoprotein TAG fatty acids, which included serum NEFA, newly made fatty acids, and dietary fatty acids. The key contributions of this work are that it (a) clearly demonstrated the dominance of serum NEFA in liver TAG pools in NAFLD; (b) clarified the effect of insulin resistance manifested through increased rates of DNL that fail to respond to nutritional state; (c) expanded

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**Table 1**

Clinical and laboratory values in NAFLD patients

<table>
<thead>
<tr>
<th>Subject characteristics (n = 9)</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>108.2 ± 17.0</td>
<td>85.8–136.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>36 ± 5</td>
<td>29–44</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>44.7 ± 11.1</td>
<td>30.2–55.9</td>
</tr>
<tr>
<td>Visceral fat (cm²)</td>
<td>231 ± 50</td>
<td>192–324</td>
</tr>
<tr>
<td>Subcutaneous fat (cm²)</td>
<td>496 ± 178</td>
<td>213–723</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>68 ± 34</td>
<td>63–186</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>102 ± 41</td>
<td>29–147</td>
</tr>
</tbody>
</table>

**Fasted** | **Fed** | **P-value** |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum insulin (pmol/l) A</td>
<td>24.8 ± 12.9</td>
<td>117.6 ± 131.4</td>
</tr>
<tr>
<td>Serum glucose (mmol/l) A</td>
<td>5.6 ± 0.8</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>NEFA (mmol/l) A</td>
<td>0.55 ± 0.33</td>
<td>0.34 ± 0.24</td>
</tr>
<tr>
<td>tTRL TAG (mmol/l) A</td>
<td>1.7 ± 1.8</td>
<td>2.2 ± 2.3</td>
</tr>
<tr>
<td>EE (kcal/kg/min) B</td>
<td>0.012 ± 0.002</td>
<td>0.014 ± 0.002</td>
</tr>
<tr>
<td>NPRQ B</td>
<td>0.83 ± 0.07</td>
<td>0.84 ± 0.07</td>
</tr>
<tr>
<td>Fat oxidation (mg/kg/min) B</td>
<td>0.58 ± 0.29</td>
<td>0.55 ± 0.32</td>
</tr>
<tr>
<td>Glucose oxidation (mg/kg/min) B</td>
<td>0.94 ± 0.59</td>
<td>1.02 ± 0.52</td>
</tr>
</tbody>
</table>

Data in the upper panel are baseline data obtained during screening procedure. NAFLD patients were on various medications, including medications for hypertension, hypothyroidism, and depression. One subject was being treated for diabetes. A Fasted and fed values represent the average concentrations obtained on days 2, 3, and 4 at 0645 (fasted) and 1500 (fed) for each subject. Values were calculated from data collected on day 4 of the inpatient protocol from 0600 to 0830 for fasting and from 1500 to 1530 for fed data. EE, energy expenditure; NPRQ, non-protein respiratory quotient.
the view of the liver’s diurnal control of fatty acid metabolism, with particular emphasis on the contribution of dietary fatty acids to human liver TAG pools; and (d) demonstrated for the first time to our knowledge the similarity between the fatty acid sources that contribute to hepatic TAG and lipoprotein TAG.

The first key finding of our research here was that the serum NEFA pool was the primary contributor to hepatic TAG in the fasting state and to lipoprotein TAG in both the fasting and fed states. Sanyal et al. found elevated insulin concentrations failed to suppress adipose fatty acid flux in NAFLD (13), a result that suggested that this source of fatty acids would be important to the development of fatty liver. Indeed, NAFLD patients treated with insulin-sensitizing agents show marked improvement in their condition (28, 30, 31). Given that the majority of liver fatty acids were derived from the serum NEFA pool, therapeutic strategies should continue to focus on the reduction of adipose tissue fatty acid flux. The second key finding of our research was that DNL was elevated in the fasting state and failed to increase postprandially. In the fasting state, the hepatic use of dietary fatty acids became reduced and the deficit was made up by adipose tissue-derived fatty acids. It is well known that postprandially, dietary fatty acids spill over into the plasma NEFA pool (35), and we have shown here that during fasting, the hepatic use of dietary fatty acids became reduced and the deficit was made up by adipose tissue–derived fatty acids. This is not needed to detect this defect, as our NAFLD patients exhibited elevated fasting DNL and failed to appropriately increase lipogenesis postprandially when fed a meal consisting of 30% of daily energy requirements (32). We have shown here that chronic consumption of a high-carbohydrate diet (32). The healthy subjects’ lipogenesis rates tripled when measured in the fasting state after 5 days of high-carbohydrate feeding, while no change was observed in the fasting lipogenic rate of insulin-resistant subjects consuming the same diet (32). We have shown here that their use for lipoprotein TAG synthesis rose reduced and the deficit was made up by adipose tissue (by the combination of spillover and liver remnant uptake) in healthy subjects (36, 37), and our findings have confirmed that in NAFLD, this source can contribute substantially (approximately 15%) to both liver and VLDL TAG. It is likely that the dietary contribution to both TAG pools would be larger if dietary fat consumption had been higher than 30% of daily energy requirements. Data from mice in our lab confirm that this is the case in animals (19, 38). Collectively, these data suggest that the liver’s system of fatty acid usage handles fluctuations that result in changes of up to 60% of the fatty acids used for VLDL TAG synthesis from fasting to feeding.

The presence of a large amount of lipoprotein remnants in hypertriglyceridemic individuals would tend to dilute apparent DNL in labeling studies less than 6 hours in duration. The 24% DNL observed here is twice the 10% reported previously in obese, hyperinsulinemic subjects (32) and is 4–5 times the less than 5% observed in healthy populations (33, 34). We have also recently documented a pattern of significant stimulation of lipogenesis in healthy subjects after consumption of a meal, rising from less than 5% to a peak of 23% within 4 hours of both the first morning meal and lunch (23). For NAFLD patients, the absence of this diurnal pattern of DNL was suggested in the daily measurements (Figure 2B) and was confirmed with a breakfast test (Figure 2C). Schwarz and colleagues found elevations in fasting DNL in obese, hyperinsulinemic subjects consuming ad libitum (high-fat) diets and demonstrated that those subjects failed to respond in the same manner as healthy subjects when switched to chronic consumption of a high-carbohydrate diet (32). The healthy subjects’ lipogenesis rates tripled when measured in the fasting state after 5 days of high-carbohydrate feeding, while no change was observed in the fasting lipogenic rate of insulin-resistant subjects consuming the same diet (32). We have shown here that chronic consumption of a high-carbohydrate diet is not needed to detect this defect, as our NAFLD patients exhibited elevated fasting DNL and failed to appropriately increase lipogenesis postprandially when fed a meal consisting of 30% fat. The lack of postprandial stimulation suggests the liver’s lipogenic potential may be reaching a threshold in these patients.

A third key finding of our studies relates to the significant extent to which the liver manages its use of the different sources of fatty acids that flow to it throughout the day. Inspection of Figure 2 demonstrates the reciprocal nature of these fluxes, such that during fasting, the hepatic use of dietary fatty acids became reduced and the deficit was made up by adipose tissue–derived fatty acids. It is well known that postprandially, dietary fatty acids spill over into the plasma NEFA pool (35), and we have shown here that their use for lipoprotein TAG synthesis rose reduced and the deficit was made up by adipose tissue (by the combination of spillover and liver remnant uptake) in healthy subjects (36, 37), and our findings have confirmed that in NAFLD, this source can contribute substantially (approximately 15%) to both liver and VLDL TAG. It is likely that the dietary contribution to both TAG pools would be larger if dietary fat consumption had been higher than 30% of daily energy requirements. Data from mice in our lab confirm that this is the case in animals (19, 38). Collectively, these data suggest that the liver’s system of fatty acid usage handles fluctuations that result in changes of up to 60% of the fatty acids used for VLDL TAG synthesis from fasting to feeding.
The final and most novel finding of our work was a significant similarity between the contributions of fatty acid sources found in the liver TAG pools and the sources used for TAG secretion by the liver via lipoproteins. In many studies, labeling with a glycerol or fatty acid isotope has been used to investigate lipoprotein TAG synthesis rates (as reviewed previously by Baker in ref. 39 and more recently by Patterson in ref. 40), but we have found only 1 paper in the literature that combined labeling of VLDL TAG with analysis of liver tissue biopsy samples (16). Farquhar and colleagues used a fatty acid label in liver turnover studies of 4 men with normal liver TAG pool sizes (e.g., estimates between 3 and 11 g TAG/liver, equivalent to 0.4–1.0% wet weight of tissue). As only a single fatty acid label was used, no comparison could be made between the various fatty acid sources contributing to liver and lipoprotein TAG. In our study here, the similarity between the 2 TAG pools provides support for future studies in which lipoprotein fatty acid may be used as an indicator for the liver TAG fatty acid pool in order to determine the success of treatments designed to reduce hepatic TAG stores.

The fact that the rates of appearance of labeled fatty acids rose over the 5 days of study deserves some comment. We expected (16, 39) and observed recycling of fatty acids over this time frame, with the majority of recycling potentially routing through the large hepatic TAG pool (16). The serum NEFA used for VLDL TAG achieved a pseudosteady state after a single day of labeling, and this observation supports the idea of the presence of a fast hepatic turnover pool through which serum NEFAs are routed (see center droplet in liver schematic, Figure 1). The significantly higher slope of the incorporation of NEFA into lipoproteins than those for the incorporation of dietary and DNL fatty acids supports the idea of differential handling of this source in the hepatocyte. The accrual into VLDL TAG of de novo fatty acids could be due to an increase in hepatic pools (i.e., those made locally and packaged into VLDL) or due to the accrual of fatty acids synthesized in the adipose that came to the liver through the serum NEFA pool, although the latter mechanism is less likely, given the 4-fold higher presence of DNL fatty acids in VLDL than in the serum NEFA pool. The lower daily rates of accrual for dietary and DNL fatty acids over the 5 days suggest that these fatty acids enter a hepatic holding pool

Table 2
Liver function and histological evaluation of NAFLD patients

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age/gender</th>
<th>BMI (kg/m²)</th>
<th>Steatosis</th>
<th>Inflammation</th>
<th>Fibrosis</th>
<th>Liver TAG (g/g tissue)</th>
<th>% TAG labeled (%/d)</th>
<th>FSR (g/d)</th>
<th>ASR (g/d)</th>
<th>Turnover (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36/M</td>
<td>29</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0.11</td>
<td>20.4</td>
<td>5.1</td>
<td>3.9</td>
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</tr>
<tr>
<td>2</td>
<td>40/F</td>
<td>38</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0.49</td>
<td>21.6</td>
<td>5.4</td>
<td>16.1</td>
<td>59.2</td>
</tr>
<tr>
<td>3</td>
<td>36/M</td>
<td>43</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>ND</td>
<td>49.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>57/F</td>
<td>39</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0.09</td>
<td>30.0</td>
<td>7.5</td>
<td>4.4</td>
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</tr>
<tr>
<td>5</td>
<td>43/M</td>
<td>30</td>
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<td>0</td>
<td>0.05</td>
<td>56.5</td>
<td>14.1</td>
<td>4.9</td>
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</tr>
<tr>
<td>6</td>
<td>61/M</td>
<td>37</td>
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<td>0</td>
<td>0</td>
<td>0.09</td>
<td>36.4</td>
<td>9.1</td>
<td>5.3</td>
<td>35.1</td>
</tr>
<tr>
<td>7</td>
<td>31/F</td>
<td>44</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>0.04</td>
<td>63.7</td>
<td>15.9</td>
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<td>9.6</td>
<td>15.8</td>
<td>33.2</td>
</tr>
<tr>
<td>9</td>
<td>36/F</td>
<td>32</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0.16</td>
<td>40.4</td>
<td>10.1</td>
<td>9.7</td>
<td>31.6</td>
</tr>
</tbody>
</table>

Mean (± SD) 36 (5) 2.7 (0.7) 1.1 (0.8) 0.7 (0.9) 0.16 (0.15) 38.4 (15.6) 9.6 (3.8) 8.2 (5.1) 38.4 (15.6)

*Graded according to Dixon (45). Steatosis grades: 1, less than 5% of lobular parenchyma involved; 2, 5–25%; 3, 25–75%. Inflammation grades: 0, no inflammation; 1, sparse; 2, mild. Fibrosis grades: 0, normal connective tissue; 1, focal pericellular fibrosis, zone 3; 2, perivenular and pericellular fibrosis, zones 2 and 3. Liver turnover times were estimated using an assumed liver weight of 2,000 g (16) and an estimation of 5 × t1/2 for near-complete turnover of the hepatic TAG pool (50). ASR, absolute synthesis rate; FSR, fractional synthesis rate; ND, not detectable (i.e., the fat content of the liver of subject 3 was below the detection limit of the gas chromatograph, and therefore no turnover time could be estimated).
that prevents them from being in immediate communication with the pool of fatty acids used for VLDL TAG synthesis. Moreover, as the final proportions of fatty acid sources in VLDL and liver were similar, it is likely that the liver TAG pool handles these sources of fatty acids in a manner similar to that of those newly made.

A number of limitations of this study deserve mention. The first is that the duration of isotope labeling was only sufficient to account for at most 64% of the sources of liver TAG fatty acids in any one subject. The duration of 5 days was chosen from a practical standpoint as being the longest time that was feasible for patients to be absent from work to undergo continuous labeling while staying as inpatients at the General Clinical Research Center (GCRC). Second, as ethically only 1 liver biopsy could be performed, it was impossible to know where on a physiologic turnover curve the liver TAG was being sampled. Nonetheless, the estimate of hepatic TAG turnover time ($5 \times t_{1/2}$) ranged from 20 to 60 days, which was surprisingly long, given estimates of 1–2 days for liver TAG turnover in healthy subjects (16, 39, 41, 42). In contrast, for VLDL TAG, 5 days of labeling was sufficient time to allow for 100% of the fatty acids to be accounted for in 5 of the 9 subjects. In the 4 subjects still secreting unlabeled lipoprotein fatty acids at the day-5 time point, the origin of this cold pool could not be definitively identified. Visceral fatty acids can dilute the serum NEFA label that would not be accounted for when sampled peripherally. This would cause an underestimation of the contribution of serum NEFA to VLDL TAG and result in the inability to account for all VLDL fatty acids (a visceral dilution effect). However, we found a strong positive correlation between the percentage of fatty acids accounted for in VLDL TAG and those accounted for in liver TAG suggesting that the origin of unlabeled VLDL fatty acids was not due to visceral dilution but due to use of a cold (unlabeled) hepatic TAG pool. Third, our study lacked a control group, because elective liver biopsy in a control population was not an option. Thus, whether the pattern of fatty acid sources would have been similar in the livers of subjects without NAFLD is unknown. However, the change in fatty acid sources found in VLDL TAG in both the fasting and fed states was compared with that from previous studies (9, 16, 33, 34, 39, 41, 42) and recent data from our lab (23, 36, 37). An inability to account for all of the fatty acids in VLDL TAG has been observed previously in hypertriglyceridemias (9). Furthermore, given our data (23) and other data in the literature (34), the lack of increase in lipogenesis postprandially in NAFLD is striking. Finally, the goals of our study were to determine the sources of fatty acids utilized for liver and VLDL TAG synthesis in NAFLD, and measurement of TAG or particle production rates was not undertaken. However, data from Charlton et al. have suggested that lipoprotein particle secretion rate (as assessed by apoB turnover) is significantly reduced in nonalcoholic steatohepatitis (43). Furthermore, using $[^{13}C]_1$palmitate infusion, Diraizon et al. have demonstrated normal TAG secretion rates in NAFLD (18). In the context of those studies, fatty liver could be caused either by low particle secretion rates or by an amount of secreted TAG per particle that is insufficient to keep up with liver TAG synthesis. If either of these events contributed to the fatty liver observed in our patients, then elevated DNL would exacerbate the situation. Our data have shown that elevated lipogenesis provides a significant source of the fatty acids accumulating in the livers of these patients.

In summary, our data provide a quantitative description of the fatty acid sources that contributed to both the liver and lipoprotein TAG of NAFLD patients after 5 days of labeling. Efforts to treat NAFLD by improving insulin sensitivity to reduce peripheral fatty acid flux should be expanded to modulate liver lipogenesis through dietary and pharmacological therapy. With obesity as a key risk factor for NAFLD, and with the recent recognition of the ability of simple sugars to stimulate the lipogenic pathway (33), dietary therapy that advocates the consumption of complex carbohydrates deserves emphasis. One of the underlying causes of fat accumulation in NAFLD is the inability of the liver to regulate the changes in lipogenesis that should occur during the transition from the fasted to the fed state.

Methods

Subjects. Men and women scheduled for a liver biopsy for diagnosis of NAFLD were recruited by participating physicians at the clinics of Minnesota Gastroenterology (Minneapolis, Minnesota, USA). Patients were not referred for the study if alcohol consumption exceeded 20 g/wk or if they tested positive for viral hepatitis, Wilson disease, or any other possible cause of liver dysfunction. The subjects’ liver function was further assessed through measurement of $\gamma$-globulins (A, G, E, and M), $\alpha_1$-antitrypsin, $\alpha_2$-fetoprotein, ferritin, ceruloplasmin, iron, transferrin, and hepatitis A, B, and C. For the present study, potential NAFLD subjects were screened on 2 occasions to obtain a detailed biochemical and nutritional assessment. Patients were included in the study if their physician suspected they had fatty liver, if they had elevated liver enzymes upon initial screening, and if they were not in a period of active weight loss (defined as weight loss exceeding 0.5 kg/wk). There were no restrictions on gender, smoking status, age, or glucose tolerance. Women participants were studied during the luteal phase of the menstrual cycle. All subjects gave written informed consent to participate in this research study, which was approved by the Institutional Review Board of the University of Minnesota (protocol 0106M01801). All medical procedures were performed at the GCRC at Fairview University Medical Center (Minneapolis, Minnesota, USA).

Procedures. Computed tomography scans without contrast were performed on each participant to quantify visceral and subcutaneous fat mass (44). To ensure metabolic steady state, 3 days prior to the 5-day inpatient stay, subjects were instructed to consume a defined eucaloric diet. This diet was based on previously acquired food records and interviews conducted during screening. Subjects recruited were required to be maintaining their weight and to be eating diets of typical composition (55% carbohydrate, 30% fat, and 15% protein). The prescribed diets consumed during the 3-day run-in phase were matched to the subjects’ background diet; thus, the subjects were deemed to be in stable metabolic states. Energy requirements were determined by the Harris-Benedict equation with an appropriate activity factor. Subjects’ weights were recorded daily to ensure weight maintenance throughout the inpatient protocol. To trace the sources of lipoprotein and hepatic TAG fatty acids in both fasted and fed states, we began isotope infusions 4 days prior to and continued them through each patient’s scheduled liver biopsy. $[^{13}C]_1$Sodium acetate, $[^{1,2,3,4}^{13}C]_3$potassium palmitate, and $[^{1,2,3,4}H]_3$glyceryl-tripalmitin were purchased from Cambridge Isotope Laboratory and Isotec. Isotopic purity was greater than 98% for all tracers used. Deuterated tripalmitate was used to determine the contribution of dietary fatty acids to hepatic and lipoprotein TAG; unpublished data from our lab support the use of this isotope in tracking unlabeled dietary fatty acids into the chylomicron fraction (M.T. Timlin and E.J. Parks, unpublished observations). To measure the contribution of serum NEFA to lipoprotein and liver TAG, we began a constant infusion of $[^{13}C]_3$potassium palmitate at a rate of 2.5 mg/kg/min on day 1 at 1700. Fatty acid synthesis was measured using a continuous infusion of sodium $[^{1,2,3,4}C]_4$acetate (10g/d), also beginning at 1700 on day 1. The contribution of dietary TAG to liver and lipoprotein
pools was measured using [3H]glyceryl-tripalmitin (1-2 g/d), which was administered in whole food with each meal eaten at the GCRC. In both the fasted state (0645) and fed state (1500), blood was drawn intermittent-
ly over the 5-day study period and lipoproteins were isolated each day. On day 4 of the protocol, subjects underwent a meal test to determine lipid flux during the postprandial period. At 0700, a breakfast was consumed consisting of whole foods (30% fat, 55% carbohydrate, and 15% protein). Of the grams of carbohydrate provided in the meal, 3.3%, 5.6%, 8.6%, 6.2%, and 53.4% were from glucose, fructose, lactose, sucrose, and starch, respectively, with the remainder of carbohydrate weight provided by dietary fiber. A fasting blood sample was obtained prior to the meal, and blood samples were collected immediately after meal consumption for every 15 minutes until 0800 and hourly thereafter. Indirect calorimetry was per-
formed twice for 30 minutes for the assessment of substrate oxidation in the fasted and fed states using a Deltratrac II metabolic cart in the hooded mode (Sensor Medics). All blood was drawn into iced Vacutainers containing 1 mg/ml EDTA. Serum was separated immediately by centrifugation (1,500 g for 20 minutes) at 4°C, kept on ice, and separated into aliquots for various analyses. The liver biopsy occurred at the GCRC at 0800 on day 5 and was performed by the referring physician. Approximately 1 hour prior to the biopsy, patients were given an intramuscular injection of 100 mg meperidine hydrochloride (Abbott Laboratories) plus 50 mg hydroxyzine hydrochloride (American Regent). After the biopsy, 15 mg of the liver tissue was set aside for analysis of TAG sources. Histologi-
cal evaluation of all biopsies was performed by the same pathologist, and samples were graded for the presence of fat infiltration, necroinflammation, and fibrosis according to the grading system of Dixon (45).

Lipoprotein isolation, lipid extraction, and laboratory analysis. To determine whether the sources of TAG fatty acids stored in the liver were utilized for TAG exported out of the liver, we isolated lipoproteins from serum as described previously (9). Briefly, tTRLs (density < 1.006 g/ml) were isolated from serum by ultracentrifugation for 20 hours in a 50.3 Beck-
man rotor (1.3 x 10^8 g at 10°C). The tTRL fraction (upper 2 ml) was col-
clected by tube slicing. For fed-state samples, tTRLs alone were analyzed. Samples collected in the fasting state were dialyzed for 48 hours to a density of 1.21 g/ml and were subjected to further liproprotein fraction-
ation. Briefly, lipoproteins with an SN greater than 400 were separated by ultracentrifugation, and after that, the lipoproteins with an SN of 60–400 were separated. The lipoproteins with an SN of 60–400 represent a frac-
tion with large VLDL particles (diameter, 70–400 nm) that are TAG rich and have a very short half-life (46). After the addition of a lipid internal standard to the lipoprotein samples with an SN of 60–400, total lipids were extracted from plasma lipoproteins and also from liver tissue by the Folch method (47). NEFAs were extracted immediately from serum with the addition of 30:70 (vol/vol) heptane/isopropanol. Individual lipids from tissue, serum, tTRL and lipoproteins with an SN of 60–400 were sepa-
rated, and the TAG and NEFA transesterified (9), and the fatty acid methyl esters were analyzed by gas chromatography and gas chromatography/mass spectrometry (GC/MS) using palmitic acid (16:0) as the marker of fatty acid metabolites. Concentrations of glucose in serum samples were measured with a Vitros Analyzer 950 (Ortho-Clinical Diagnostics) and concentrations of insulin were determined by chemiluminescence immu-
nosay (Diagnostic Products Corporation). Serum TAG and NEFA concentrations were determined by enzymatic assay (Wako Chemicals USA Inc.) with a microtiter spectrophotometer (Model EL 340 Micro-
plate; Bio-Tek Instruments Inc.). For determination of the enrichment of label in the total dietary fat, all foods consumed on a single day were placed in a blended. The enrichment of the TAG palmitate of this com-
posite food was quantified by GC/MS and was shown to be 9.9 ± 4.9%. Lipoproteins were fractionated from serum collected at 0645 on days 2, 3, 4, and 5 for the fasting samples and at 1500 for postprandial samples. GC/MS was performed on an HP 6890 with a Mass Selective Detector HP 5973 fitted with an ETP electron multiplier (SGE Inc.) using a HP 1-25-m column, with a 25-μm inner diameter and 0.33-μm film thickness and helium as the carrier gas. Electron impact was used to selectively monitor ions with mass/charge (m/z) ratios of 270, 271, 272, 274, 300, and 301. Comparable ion peak areas between standards and biological samples were achieved by either adjustment of the vol-
ume injected or by dilution or concentration of the sample. The TAG concentrations in liver samples were determined using an HP6890 gas chromatograph (Hewlett Packard). The gas chromatograph was fitted with an automatic split-injection system and a flame-ionization detect-
or. A fused silica capillary column was used (50 m in length, internal diameter of 0.18 μm, 007-23; Quadrex Corp.), and individual fatty acids were identified by retention time and were compared with an internal standard for quantification.

Isotope methodology, calculations, and statistics. By tracking the variously labeled methyl-palmitate isotopomers (M₀, M₁, M₂, M₃, M₄, and M₅) in liver TAG and VLDL TAG using GC/MS, information regarding the sources of fatty acids used for TAG synthesis was obtained. These data are presented as the proportion of fatty acids derived from the three sources (i.e., data are presented as percentage of fatty acids in VLDL TAG from NEFAs, lipogenesis, and diet). In liver and lipoproteins, DNL was calculated using mass isotoper distribution analysis (MIDA) (48, 49). MIDA is a technique based on combinatorial probabilities, with the central principle being that the isotopic enrichment of the molecule of interest is directly dependent on the enrichment of the precursor pool used for its synthesis. Thus, the labeling pattern in the precursor pool is determined by the isotopomer pattern of the polymer. Dietary and NEFA enrichments of both liver and lipoproteins were calculated using a 5-point standard curve. Comparable ion peak areas between the stan-
dard curve and biological samples were achieved by either dilution or concentration of the sample. The RaNEFA into the serum NEFA pool was calculated using the steady-state NEFA enrichment obtained on the morning of day 2 (approximately 12 hours after the start of infusion). At later time points it was anticipated that NEFA recycling would reduce the accuracy of this measurement. The infusate composition and enrichment were also analyzed by GC and GC/MS and the calculations were adjusted for the amount of unlabeled fatty acids present that were derived from less than 100% isotopic purity and from fatty acids present on the albu-
min used in the infusion. The sources of serum NEFA as shown in Fig-
ure 2A are adipose, DNL, and diet, although it should be pointed out that dietary fatty acids in the NEFA pool are designated as originating in chylomicron spillover. Our methodology cannot discern whether these fatty acids actually entered adipose and were rereleased into the serum NEFA pool. Furthermore, newly made fatty acids in the serum NEFA pool could have been synthesized de novo in either adipose tissue (and released after TAG lipolysis) or liver (and derived from VLDL spillover). The RaNEFA discussed here takes into account the input of unlabeled dietary fatty acids that spilled over into the serum NEFA pool. Spillover fatty acids were subtracted from the NEFA concentration, then adipose contribution was then calculated using this equation for corrected M₄.

Equation 1

\[
\% [\text{M}_x\text{Hexadecanoate in NEFA}] = \frac{[\text{d}_x\text{Hexadecanoate in NEFA}] - \% [\text{d}_x\text{Hexadecanoate in dietary TAG}] + \% [\text{d}_x\text{Hexadecanoate in NEFA}] - \% [\text{d}_x\text{Hexadecanoate in dietary TAG}] + \% [\text{d}_x\text{Hexadecanoate in NEFA}]}{1 - \% [\text{d}_x\text{Hexadecanoate in dietary TAG}]}\]

where d indicates deuterium.
The percentage of VLDL TAG palmitate derived only from adipose tissue release is calculated by Equation 2.

**Equation 2**

\[
\% \left[ ^{13}C \right]_{\text{hexadecanolate in VLDL TAG}} \text{corrected } M_s
\]

The percentage of VLDL TAG from diet-derived NEFA (the spillover pathway) is calculated by Equation 3.

**Equation 3**

\[
\frac{\% \left[ ^{13}C \right]_{\text{hexadecanolate in NEFA}}}{\% \left[ ^{13}C \right]_{\text{hexadecanolate in NEFA}}} \times \frac{\% \left[ ^{13}C \right]_{\text{hexadecanolate in VLDL TAG}}}{\% \left[ ^{13}C \right]_{\text{hexadecanolate in dietary TAG}}}
\]

Among all subjects, it was observed that the ratio of \([^{13}C]_{\text{palmitate}}\) to \([^{13}C]_{\text{palmitate}}\) was always greater in VLDL TAG than in the serum NEFA pool. This additional source of dietary fatty acids must be from chylomicron-remnant TAG that entered the liver either directly via remnant receptor uptake or indirectly via transfer to HDL, or via lipolysis at the hepatocyte. Thus, given Equation 3, the percentage of VLDL TAG derived from hepatic uptake of chylomicron-remnant TAG is calculated by subtracting the portion of dietary fatty acids derived via the spillover pathway from the total contribution of dietary fatty acids to VLDL TAG.

**Equation 4**

\[
\frac{\% \left[ ^{13}C \right]_{\text{hexadecanolate in VLDL TAG}}}{\% \left[ ^{13}C \right]_{\text{hexadecanolate in dietary TAG}} - \text{Equation 3}}
\]

Liver turnover data were calculated using an assumed liver weight of 2,000 g and an estimation of 5 to 10% for near-complete turnover of the hepatic TAG pool (50). Differences in source contribution over time were analyzed using repeated-measures analysis of variance. Statistical differences between fasting and feeding and between sources of VLDL TAG palmitate and liver TAG palmitate were determined by paired Student’s t tests using Statview (version 5.0.1.; SAS Institute Inc.). Correlations were analyzed using simple regression. A P value of less than 0.05 was considered significant.

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