Hepatic VLDL assembly is disturbed in a rat model of nonalcoholic fatty liver disease: is there a role for dietary coenzyme Q?

Ainara Cano,2 Fiorella Ciaffoni,3 Ghada M. Safwat,3 Patricia Aspichueta,2 Begoña Ochoa,2 Elena Bravo,3 and Kathleen M. Botham1

1Department of Veterinary Basic Sciences, Royal Veterinary College, London, United Kingdom; 2Department of Physiology, Faculty of Medicine and Dentistry, University of Basque Country, Leioa, Spain; and 3Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore Sanità, Rome, Italy

Submitted 19 March 2009; accepted in final form 15 July 2009

Cano A, Ciaffoni F, Safwat GM, Aspichueta P, Ochoa B, Bravo E, Botham KM. Hepatic VLDL assembly is disturbed in a rat model of nonalcoholic fatty liver disease: is there a role for dietary coenzyme Q (CoQ)? J Appl Physiol 107: 707–717, 2009. First published July 16, 2009; doi:10.1152/japplphysiol.00297.2009.—The overproduction of very-low-density lipoprotein (VLDL) is a characteristic feature of nonalcoholic fatty liver disease (NAFLD). The aim of this study was to use a high-fat diet-induced model of NAFLD in rats to investigate 1) the influence of the disease on hepatic VLDL processing in the endoplasmic reticulum and 2) the potential modulatory effects of dietary coenzyme Q (CoQ). Rats were fed a standard low-fat diet (control) or a diet containing 35% fat (57% metabolisable energy). After 10 wk, high-fat diet-fed animals were divided into three groups: the first group was given CoQ9 (30 mg·kg body wt−1·day−1 in 0.3 ml olive oil), the second group was given olive oil (0.3 ml/day) only, and the third group received no supplements. Feeding (3 high-fat diets and the control diet) was then continued for 8 wk. In all high-fat diet-fed groups, the content of triacylglycerol (TG) and cholesterol in plasma VLDL, the liver, and liver microsomes was increased, hepatic levels of apolipoprotein B48 were raised, and the activities of microsomal TG transfer protein and acyl CoA:cholesterol acyltransferase were reduced. These findings provide new evidence indicating that VLDL assembly and the inherent TG transfer to the endoplasmic reticulum are altered in NAFLD and suggest a possible explanation for both the overproduction of VLDL associated with the condition and the disease etiology itself. Dietary CoQ caused significant increases in apolipoprotein B mRNA and microsomal TG levels and altered the phospholipid content of microsomal membranes. These changes, however, may not be beneficial as they may lead to the secretion of larger, more atherogenic VLDL.

apolipoprotein B; triacylglycerol; microsomal triacylglycerol transfer protein; microsomal membrane lipids; hepatic steatosis; very-low-density lipoprotein

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) is the most commonly occurring liver disorder in the world. It occurs when lipid accumulates inside liver cells and is strongly associated with obesity, insulin resistance, diabetes type 2, and metabolic syndrome (1). The simple hepatic steatosis seen in NAFLD may progress into more serious liver disease, including nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and, more rarely, hepatocellular carcinoma and liver failure (1, 10). NAFLD is present in 70–90% of individuals with obesity and diabetes type 2, and in patients with metabolic syndrome the condition is more likely to progress to a more severe form (43).

It is well known that pathologies such as obesity and insulin resistance, which make up metabolic syndrome, are associated with an increased risk of cardiovascular disease, but recent evidence has suggested that NAFLD itself is an independent risk factor (43). Possible mechanisms by which the condition may contribute to premature atherosclerosis development include altered liver lipoprotein metabolism, particularly in the postprandial phase (2, 11, 43), and increased oxidative stress, which results from hepatic lipid accumulation and insulin resistance (19).

Under normal physiological conditions, the liver stores fat when the supply exceeds the immediate demand and secretes it in the form of very-low-density lipoprotein (VLDL) when energy for peripheral tissues is needed. For VLDL synthesis, triacylglycerol (TG) is mobilized from cytosolic droplets by lipolysis followed by reesterification by diacylglycerol acyltransferase (DGAT)2, and the resulting product may be transferred by the action of microsomal TG transfer protein (MTP) into the endoplasmic reticulum (ER), where it enters the VLDL precursor pathway (18). An alternative fate for the TG product, however, is return to the cytosol by a process that is enhanced by insulin and results in the suppression of VLDL secretion (18). NAFLD is associated with hypertriglyceridemia caused by the overproduction of large, TG-rich VLDL (2, 47). This effect may be a consequence of hepatic insulin resistance (3, 47), but the mechanisms underlying these disturbances in the processing of VLDL components in the ER and the subsequent secretion of the particles are not well understood.

The oxidative stress associated with hepatic lipid accumulation and insulin resistance depletes intracellular concentrations of natural cellular antioxidants (19). Coenzyme Q (CoQ) (also called ubiquinone) is the only lipid soluble antioxidant that is synthesized endogenously (6). It is found in all membranes, and the reduced form (ubiquinol) acts in cells and tissues to reduce lipid peroxidation (6). CoQ is a highly efficient antioxidant in cells for a number of reasons: the reduced form can be regenerated after oxidation in all subcellular compartments, it is located in membranes, and it is present in relatively high concentrations compared with other lipid-soluble antioxidants (6). In NAFLD, however, fat accumulation and lipid peroxidation may cause changes in the physical and chemical properties of liver membranes, and hepatic concentrations of CoQ may be altered (21). Thus, it might be expected that NAFLD and insulin resistance would cause disturbances in CoQ concentrations in the liver (16, 48). Despite the relationship between NAFLD and oxidative stress, studies of the effects of dietary antioxidant supplements, including vitamins C and E, N-acetylcysteine, and reduced glu-
tathione, have given inconclusive results (28). As far as we are aware, however, the effects of dietary CoQ on hepatic VLDL processing in the ER in NAFLD have not been investigated previously in either humans or experimental animals.

To study the effects of NAFLD on the cellular events involved in VLDL assembly and secretion, it is necessary to use an animal model. Rodents with a genetic defect or given a diet deficient in choline or methionine have been used most commonly in previous studies (26), but these models have deficiencies in mimicking the human disease. Recently, therefore, methods have been developed to induce NAFLD by caloric overconsumption (27, 42, 49), which resembles the conditions that give rise to the disease in the human population. Feeding rats high-fat liquid diets (71–77% energy from fat) (27, 49) or a solid high-fat diet (58% energy from fat) (42) has been found to induce the characteristic features of NAFLD, including hepatic steatosis, liver damage, and insulin resistance. The first aim of this study was to investigate the influence of NAFLD on hepatic VLDL processing in the ER using a high-fat diet (57% of energy from fat) (42) to induce the condition in rats. We (40) have previously shown that this diet causes insulin resistance, hypertriglyceridemia, hepatic steatosis, and liver damage and thus provides a good model of the early stages of the disease; however, this study (40) provided no information about the effects of the disease on the assembly of VLDL in the liver. In the present investigation, the effects on plasma VLDL concentrations, liver lipids and apolipoprotein B (apoB) content, microsomal lipid levels, acyl CoA:cholesteryl ester transferase (ACAT) and MTP activity, and mRNA expression of apoB, MTP, and DGAT2 and other related proteins were determined. The second aim was to determine whether hepatic VLDL processing in NAFLD is modulated by dietary CoQ. Rats were fed a standard low-fat diet or a diet containing 57% of the metabolizable energy as fat for 18 wk. For the investigation of the effects of CoQ, in the last 8 wk of the study, subgroups of rats on the high-fat diet were given the compound dissolved in olive oil or olive oil only. Although in humans the side chain of CoQ consists of 10 isoprenes (CoQ10, the form generally used in clinical trials), in rats and mice the major CoQ form has 9 isoprenes (CoQ9) (7), and for this reason a reduced and stabilized form of CoQ9 (CoQ9 monomethyl ether) was used in the present study. CoQ9 and CoQ10 are equally efficient antioxidants and redox carriers and have similar distributions in tissues; thus, the minor difference in side chain length is not thought to be functionally important (6).

MATERIALS AND METHODS

Materials. The monomethyl ether of CoQ9H2 [2,3,4-trimethoxy-6-methyl-5-(all trans)-nonaprenylphenol, kindly supplied by Dr. Giorgio Borioni (Synth-Active, Rome, Italy)] used as the source of CoQ9 was synthesized from solanesol (all-trans-nonaprenol) (9).

Animals and diets. Male Wistar rats (195 ± 7 g) obtained from Harlan Italy (S. Pietro al Natisone, Italy) were housed individually at a temperature of 22 ± 1°C with a 12:12-h light-dark cycle and allowed food and water ad libitum. Experiments were carried out in accordance with the guidelines of the European Community Council for animal care and use, and all procedures performed conformed strictly with Directive 86/609/EEC. Experiments were also approved by the Animal Care Committee of the Istituto Superiore di Sanita.

Initially, rats were divided into two groups fed either a standard low-fat rat chow diet (control diet, 7 rats) or a high-fat diet (16 rats) (Mucedola, Settimo Milanese, Italy). After 10 wk, the high-fat-diet fed group was divided into three subgroups, and feeding was continued for a further 8 wk. One subgroup (6 rats) received reduced stabilized CoQ9 (30 mg·kg body wt⁻¹·day⁻¹) in 0.3 ml extra virgin olive oil by gavage (high-fat + CoQ diet), the second subgroup (6 rats) was given olive oil (0.3 ml/day) only by the same route (high-fat + olive oil diet), and the third subgroup (4 rats) received no supplements (high-fat diet). Table 1 shows the composition of the diets. The control diet contained 4.3% fat (10% of the metabolizable energy), and the high-fat diet contained 35% fat (31.6% saturated fat and 3.2% unsaturated fat, 57% of the metabolizable energy).

At the end of the experiments (after 18 wk), all rats were killed after being fasted overnight. Blood samples collected in heparinized tubes via heart puncture with the rats under terminal anesthesia were centrifuged (3,500 rpm for 15 min at 6°C) to obtain plasma. Livers were excised, washed with cold physiological saline solution (0.9%), dried, and stored at −80°C until required for analysis.

Separation of plasma VLDL. VLDL was isolated from 0.5 ml plasma by step ultracentrifugation according to Friedewald et al. (17). VLDL (density: <1.006 g/ml) fractions were dialyzed overnight against PBS before analysis of the lipid content.

Determination of microsomal lipid content and ACAT activity. Liver samples (500 mg) in ice-cold Tris·HCl buffer (50 mM) containing sucrose (0.25 M), EDTA (1 mM), and sodium azide (0.02% wt/vol) at pH 7.4 were homogenized using a Potter Elvejhem homogenizer, and microsomes were isolated by step centrifugation as described by Ruiz and Ochoa (39). Lipids were extracted from the pellet by the method of Bligh and Dyer (8), and the microsomal content of TG, unesterified cholesterol (UC), cholesteryl ester (CE), and the major phospholipids was determined by TLC followed by image analysis as previously described (39). ACAT activity was assayed under saturating exogenous cholesterol conditions using [1-14C]oleoyl-CoA as previously described (24).

Determination of MTP TG transfer activity. Liver samples (100 mg) in ice-cold Tris·HCl buffer (1 mM, pH 7.6) containing EGTA (1 mM), MgCl2 (1 mM), and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) were homogenized using a Polytron homogenizer. Homogenates were centrifuged (7,437 g, 4°C) and MTP activity was measured in the supernatants using a fluorescence assay (Chylos, Woodbury, NY) (4) performed in triplicate. MTP TG transfer activity was calculated as the percentage of

Table 1. Composition of the control and high-fat diets

<table>
<thead>
<tr>
<th>Component</th>
<th>High fat diet</th>
<th>Control diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolizable energy, kcal/kg</td>
<td>5,200</td>
<td>2,652</td>
</tr>
<tr>
<td>Metabolizable energy from fat, %</td>
<td>10</td>
<td>57</td>
</tr>
<tr>
<td>Total protein, %</td>
<td>19</td>
<td>26</td>
</tr>
<tr>
<td>Total fat, %</td>
<td>35</td>
<td>43</td>
</tr>
<tr>
<td>Total fiber, %</td>
<td>6.5</td>
<td>5</td>
</tr>
<tr>
<td>Total ash, %</td>
<td>4.5</td>
<td>4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>332</td>
<td></td>
</tr>
<tr>
<td>Lard</td>
<td>316</td>
<td>19</td>
</tr>
<tr>
<td>Corn starch</td>
<td>89</td>
<td>298</td>
</tr>
<tr>
<td>Casein</td>
<td>258</td>
<td>190</td>
</tr>
<tr>
<td>Cellulose</td>
<td>65</td>
<td>47</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td>Minerals</td>
<td>58</td>
<td>43</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>162</td>
<td>33</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>13</td>
<td>10</td>
</tr>
</tbody>
</table>
transfer per hour per milligram of protein after correction using blank values.

Determination of liver apoB content. Liver samples (60 mg) in ice-cold buffer (pH 8.0) containing Tris (50 mM), NaCl (150 mM), Nonidet P-40 (1.0%), sodium deoxycholate (0.5%), SDS (0.1%), and protease inhibitor cocktail were homogenized using a Polytron homogenizer. After centrifugation (12,000 g for 20 min at 4°C), pellets were collected, and samples (250 μg protein) were resolved by SDS-PAGE at 150 V for 1.5 h. Proteins were then transferred to polyvinylidene difluoride membranes by semidyse transference (3.5 h at 40 V), and apoB48 and apoB100 were detected by immunoblot analysis using a goat anti-human apoB antibody (cat. no. SC-11795, Santa Cruz Biotechnology, Santa Cruz, CA) and a peroxidase-coupled donkey anti-goat IgG (cat. no. SC-2304, Santa Cruz Biotechnology) as described by Vine et al. (46). ApoB bands were visualized using ECL (ECL-Advance, Amersham Biosciences) and quantified using QuantityOne software (Bio-Rad Laboratories, Herts, UK). Results are expressed as densities of apoB48 and apoB100 bands in the samples relative to that of a reference sample assayed in all the gels.

Determination of mRNA expression. The relative transcript abundance of apoB, MTP, DGAT1, DGAT2, carboxylesterase (CES)3, arylacetamide deacetylase (AADA), and ADP ribosylation factor (ARF)1 was determined by quantitative PCR. Total RNA was extracted from the liver using the GenElute Mammalian Total RNA kit (Sigma, Dorset, UK) according to the manufacturer’s instructions. First-strand cDNA synthesis was carried out using a Reverse Transcription System kit (Promega, Madison, WI), and quantitative PCR was performed in a DNA Engine Opticon 3 Light cycler (MJ Research, GMI, Ramsey, MN) using SYBR Green Jump Start Taq Ready Mix (Sigma). The forward and reverse oligonucleotide primers used for the tested genes as well as the housekeeping genes (β-actin, albumin, and β2-microglobulin) are shown in Table 2. PCR conditions were as follows: 1) denaturation at 94°C for 2 min followed by 37 cycles of 94°C for 15 s, annealing at 56°C for 1 min, a final extension of 72°C for 1 min, and a melting curve program (60–95°C, heating rate: 0.2°C/s). The threshold cycle values were determined from the melting curve data. The relative transcript abundance of each gene was normalized using β-actin, albumin, and β2-microglobulin as housekeeping genes. Normalization was performed using the Software Application GeNorm (45). According to GeNorm, β-actin, albumin, and β2-microglobulin were suitable genes for normalization for the present study.

Other analytical methods. The lipid content of plasma VLDL and liver lipids after extraction with chloroform-methanol [2:1 (vol:vol)] was determined using kits for TG and cholesterol (Alpha Laboratories, Eastleigh, UK). Proteins were measured in liver microsomes and MTP and apoB samples using bicinchoninic acid (Thermo Fisher Scientific, Rockford, IL).

Significance limits were calculated using one-way ANOVA followed by the Bonferroni’s multiple-comparison test (multiple groups). In cases where there was no significant difference between the three high-fat diet-fed groups, the data were combined and significance limits versus the control diet-fed group were calculated using Student’s t-test. It is important to note, however, that comparisons made with the combined data have some limitations. Correlations were calculated using Pearson’s correlation coefficient.

RESULTS

Plasma VLDL and liver lipids. TG and total cholesterol (TC) levels in plasma VLDL isolated from rats fed the four different diets are shown in Fig. 1. There was an increase of ~3.5- to 4.5-fold in VLDL TG (Fig. 1A) and ~2.5-fold in VLDL cholesterol (Fig. 1B) in all three high-fat diet-fed groups compared with the controls (Fig. 1). The TG-to-TC ratio in VLDL was also raised in all high-fat diet-fed groups, although the effect in rats fed the high-fat + CoQ diet was the most marked. In the liver, when data from all the high-fat diet-fed groups were combined, there was a significant increase in both TC (P < 0.01) and TG (P < 0.001). In addition, TG levels were significantly raised by 2.5- to 3-fold by all the individual high-fat diets (Table 3). Liver TC content, however, was significantly increased only in the high-fat + olive oil diet-fed group (Table 3).

Liver apoB content and ACAT and MTP activity. Feeding of the high-fat diet (all high-fat diet-fed groups combined vs. the control diet) caused a significant increase (20–50%) in the hepatic content of apoB48 (P < 0.05) but not apoB100 (Fig. 2). However, in the individual groups, significance compared with the control group was reached only in the high-fat + CoQ diet-fed group (+50.8%; Fig. 2B).

There was a marked inhibition of ACAT activity in all the high-fat diet-fed groups (~79–90%; Fig. 3A). The TG transfer activity of MTP in liver microsomes was decreased in rats fed the high-fat diets (~79–90%) showing significant changes (Fig. 3B). There was no significant correlation between MTP activity and liver content of either apoB48 or apoB100 (data not shown). However, there was a highly significant negative correlation between liver TG content and MTP TG transfer activity (Fig. 4A). There was a particularly strong negative correlation between these param-

---

Table 2. Primers used for quantitative PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Product</th>
<th>GenBank Code/Identifier</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>β-Actin</td>
<td>NM_031144</td>
<td>5′-GGGAAAATGTTGGCTGAGATTT-3′</td>
<td>5′-GGGCGATGTGGCATCTC-3′</td>
</tr>
<tr>
<td>Alb</td>
<td>Albumin</td>
<td>NM_13426</td>
<td>5′-ATCTTGAAGGTTGGAAAGTG-3′</td>
<td>5′-TTTCCAGACGACCGACTA-3′</td>
</tr>
<tr>
<td>B2m</td>
<td>β2-Microglobulin</td>
<td>NM_012512</td>
<td>5′-ATCTGAGGGTGGAACCTG-3′</td>
<td>5′-TGACGGATTGTTCTTGGT-3′</td>
</tr>
<tr>
<td>Apob</td>
<td>ApoB</td>
<td>NM_019287</td>
<td>5′-ATGTCCTGCACTCCACATTTA-3′</td>
<td>5′-AAAGAACACAAAGAATGCTG-3′</td>
</tr>
<tr>
<td>Mtp</td>
<td>MTP</td>
<td>NM_00110772</td>
<td>5′-AGAACCAATGTTGAGAGAC-3′</td>
<td>5′-AGTGAAGGATTTGAGCCCAAATGCT-3′</td>
</tr>
<tr>
<td>Arf1</td>
<td>ARF1</td>
<td>NM_022518</td>
<td>5′-GAGAACCAATGTTGAGAGAC-3′</td>
<td>5′-CTATCAACGCTGGGAAACAG-3′</td>
</tr>
<tr>
<td>Ces3</td>
<td>CES3</td>
<td>BC061789</td>
<td>5′-TCCCGCTTTATGCCTGGTT-3′</td>
<td>5′-CCAGACGCTGAAGACCGAA-3′</td>
</tr>
<tr>
<td>Aadac</td>
<td>AADA</td>
<td>BC081143</td>
<td>5′-GTACGAGCACACTCCACGACG-3′</td>
<td>5′-AGTGTTCGCCACACCTCTG-3′</td>
</tr>
<tr>
<td>Dgat1</td>
<td>DGAT1</td>
<td>NM_053437</td>
<td>5′-AAAGTGGTGAAAGAAAAAGCA-3′</td>
<td>5′-AACGCTATCCGAGAACCTGA-3′</td>
</tr>
<tr>
<td>Dgat2</td>
<td>DGAT2</td>
<td>NM_00101234</td>
<td>5′-CTGACCAGCATCTGGATTG-3′</td>
<td>5′-TVTTTTCCAAAAGAAGACA-3′</td>
</tr>
</tbody>
</table>

ApoB, apoprotein B; MTP, microsomal triglyceride transfer protein; ARF1, ADP ribosylation factor 1; CES3, carboxylesterase 3; AADA, arylacetamide deacetylase; DGAT, diacylglycerol O-acyltransferase.
MTP mRNA concentrations showed a similar pattern to that found for MTP activity (Fig. 3B), although in this case the changes did not reach significance (Fig. 3B). High-fat feeding caused an increase in DGAT2 mRNA levels ($P < 0.05$; all high-fat diet-fed groups combined vs. the control diet) with the high-fat (+50.5%) and high-fat + olive oil diets (+55.7%) but not the high-fat + CoQ diet showing significant increases (Fig. 5C). The test diets had no significant effect on the levels of mRNA for other genes involved in VLDL assembly, including DGAT1, AADA, CES3, and ARF1 (data not shown).

**Microsomal lipids.** Figure 6 shows the TG, UC, and CE content of liver microsomes from rats fed the four different diets. Microsomal TG levels were increased by high-fat feeding, and the increase was particularly marked in the high-fat + CoQ diet-fed group (3.5-fold), whereas the changes observed in the high-fat and high-fat + olive oil diet-fed groups did not reach significance (Fig. 6A). TG concentrations in liver microsomes from rats fed the high-fat + CoQ diet were also significantly higher than those given the high-fat + olive oil diet (1.8-fold). There was also a significant negative correlation between microsomal TG levels and MTP activity (Fig. 7).

High-fat feeding also caused a rise in microsomal UC content in rats fed the high-fat diet only compared with those fed the control (+45.9%), high-fat + CoQ (+53%), and high-fat + olive oil (+60%) diets (Fig. 6B). In addition, the TG-to-UC ratio was significantly increased in the high-fat + CoQ diet-fed group compared with the control (3.7-fold), high-fat (2.4-fold), and high-fat + olive oil (1.8-fold) diet-fed groups. Microsomal CE levels were not significantly changed by any of the test diets (Fig. 6C).

The influence of the test diets on the microsomal content of the major phospholipids is shown in Fig. 8. Sphingomyelin (SM) levels were significantly reduced in the high-fat + CoQ diet-fed group compared with both control (-41.5%) and high-fat (-52.6%) diet-fed rats (Fig. 8A). Microsomal phosphatidylinositol (PI) concentrations, on the other hand, were significantly higher in the animals given the high-fat (+97.3%) and high-fat + CoQ (+125%) diets than in those fed the high-fat + olive oil diet (Fig. 8B). Levels of other phospholipids, including phosphatidylcholine, phosphatidylethanolamine, phosphatidyserine, and cardiolipin, were not significantly changed by any of the test diets (Fig. 8, C–F). There were also no significant changes in the phosphatidylcholine-to-phosphatidylethanolamine ratio (Fig. 8G); however, the ratio of SM to UC was decreased in the high-fat + CoQ diet-fed group compared with the control group (-38.7%; Fig. 8H).
**DISCUSSION**

NAFLD is a common condition in developed countries, being found in 25–30% of the general population and in >75% of individuals suffering from obesity, type 2 diabetes, and metabolic syndrome (1, 10, 43). Recent evidence has suggested that the disease is associated with an increased risk of atherosclerosis and mortality from cardiovascular disease, not only because of its relationship to the features of metabolic syndrome but as an independent risk factor in its own right (43).

One factor linking NAFLD to premature atherosclerosis development is dyslipidemia, including hypertriglyceridemia due to the overproduction of large VLDL, which are metabolized to strongly atherogenic small dense LDL (3, 43, 47). It has been proposed that one of the functions of VLDL secretion is to prevent fat accumulation in the liver, and steatosis does indeed occur in experimental models where VLDL formation is disrupted (30). Paradoxically, however, in NAFLD the liver produces too much VLDL, but although the cellular events regulating VLDL overproduction have been widely studied in genetic and diet-induced (e.g., fructose feeding) animal models of insulin resistance (5), in high-fat diet-induced NAFLD the changes that occur in the microsomal processing of VLDL remain unclear.

### Table 3. Liver lipid content

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Control</th>
<th>High fat</th>
<th>High fat + CoQ</th>
<th>High fat + olive oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerol, μmol/g liver</td>
<td>20.2±4.7</td>
<td>57.2±6.3*</td>
<td>52.0±9.3*</td>
<td>66.3±5.5†</td>
</tr>
<tr>
<td>Cholesterol, μmol/g liver</td>
<td>83.8±2.5</td>
<td>105.7±6.9</td>
<td>95.4±6.4</td>
<td>109.3±7.0*</td>
</tr>
</tbody>
</table>

Data are means ± SE. Rats were fed a standard low-fat diet (control; n = 7) or a high-fat diet for 18 wk in total. After 10 wk, one group on the high-fat diet received coenzyme Q9 (CoQ9; 30 mg·kg body wt·day⁻¹) in 0.3 ml extra virgin olive oil by gavage (high fat + CoQ; n = 6), the second group was given olive oil (0.3 ml/day) only by the same route (high fat + olive oil; n = 6), and the third group received no supplements (high fat; n = 4). The liver content of lipids was assayed after lipid extraction and separation as described in MATERIALS AND METHODS. *P < 0.05 and †P < 0.001 vs. the control group.

---

**Fig. 2.** Rats were fed a standard low-fat diet (control) or a high-fat diet for 18 wk in total. After 10 wk, rats fed the high-fat diet were divided into the HF + CoQ, HF + olive oil, and HF subgroups. At the end of the experiment, the livers were excised, and apolipoprotein B (apoB)48 and apoB100 levels were determined by immunoblot analysis. A: apoB48 and apoB100 bands from two different rats in each diet-fed group (250 μg loaded/lane). The reference sample (Ref) is shown on the right. B: quantification of band densities. Data are means ± SE; n = 4 animals in the HF group, 5 animals in the HF + olive oil group, 6 animals in the HF + CoQ group, and 7 animals in the control group. *P < 0.05 vs. the control group.

**Fig. 3.** Rats were fed a standard low-fat diet (control) or a high-fat diet for 18 wk in total. After 10 wk, rats fed the high-fat diet were divided into the HF + CoQ, HF + olive oil, and HF subgroups. At the end of the experiment, the livers were excised, and the activities of acyl CoA:cholesterol acyltransferase (ACAT; A) and microsomal TG transfer protein (MTP; B) were determined. Data are means ± SE; n = 3 animals in the HF group for ACAT activity, 4 animals in the HF group for MTP activity, 6 animals in the HF + CoQ group, 6 animals in the HF + olive oil group, and 7 animals in the control group. *P < 0.05 and **P < 0.001 vs. the control group.
NAFLD has been found to be associated with the overproduction of large VLDL in humans (2, 30, 44, 47). As expected, in the present study, plasma VLDL TG and cholesterol concentrations were elevated in rats fed the high-fat diets (Fig. 1). There was, however, a rise in the TG-to-TC ratio (1.7- to 2-fold), indicating that the particles were larger. There was also a marked rise in the TG content (3-fold) of the livers of animals fed all the high-fat diets and a more modest rise (15–30%) in liver cholesterol levels (Table 3). In a previous study, we have shown that rats fed the high-fat diet were insulin resistant, having a homeostatis model assessment of insulin resistance of approximately sixfold higher than control rats (40). Thus, the high fat diet-fed rats appear to show the hepatic steatosis and increased secretion of large VLDL associated with NAFLD and insulin resistance (2). Since we did not measure hepatic VLDL secretion directly, however, the possibility that the raised plasma levels may be due to impaired clearance of the larger VLDL particles cannot be ruled out.

The assembly and secretion of VLDL in liver cells are thought to occur in two steps. First, a precursor particle forms when apoB and TG are brought together in the membrane of the rough ER by a process enhanced by MTP. Second, precursor particle fuses with a larger TG-rich particle to form the nascent lipoprotein (18). Hussain et al. (23) recently proposed that in the ER membrane, the NH2-terminal end of apoB acquires neutral lipids to form nucleation sites, the number of which may be increased by both increased TG synthesis and MTP TG transfer activity. A second activity of MTP, which promotes the transfer of phospholipids, is then required for the release (or desorption) of the particles from the membrane. The TG in the TG-rich particle is synthesised by DGAT2 after lipolysis of cytosolic TG by arylacetamide deacetylase or TG hydrolase and is then either translocated into the ER lumen by MTP to contribute to VLDL assembly or returned to the cytosol in a pathway promoted by insulin (18). Thus, under normal conditions, insulin downregulates VLDL secretion. In hepatic steatosis, however, insulin fails to suppress secretion, and there is an overproduction of large VLDL (3, 18).

Reduced MTP activity has been found to be associated with fatty livers in patients with abetalipoproteinemia and hepatitis.
C and in subjects given MTP antagonists (12, 22, 31, 36), and Sugimoto et al. (41) suggested that its decreased activity is an initiating factor in alcoholic liver steatosis in rats. Our experiments show that in high-fat diet-induced NAFLD, MTP TG transfer activity (Fig. 3B) is lowered and that it is negatively correlated with both liver (Fig. 4) and microsomal TG concentrations (Fig. 7). We conclude, therefore, that reduced MTP activity contributes to hepatic TG accumulation in NAFLD, and, as far as we are aware, this is the first report of such an effect in this condition. In contrast to previous findings in fatty liver disease caused by alcohol intake or hepatitis C virus infection (31, 41), however, we found no significant change in MTP mRNA levels, suggesting that the change in the activity of the enzyme is a posttranslational effect in our model.

Iqbal et al. (25) have previously shown that liver UC levels are increased in mice with a conditional deletion of the MTP gene and that this was due to inhibition of CE synthesis. We also found an inhibition of the activity of ACAT, the enzyme responsible for cholesterol ester synthesis (38) together with reduced MTP activity after rats were fed the high-fat diet (Fig. 3), and this may explain the increase in microsomal UC levels we observed in the high-fat diet-fed NAFLD group (Fig. 6B). This effect was not found in animals fed the high-fat + CoQ and high-fat + olive oil diets, but microsomal SM concentrations were lower in these rats than in those fed the high-fat diet only (Fig. 8), suggesting that there may be an effect on membrane physiology/biochemistry. Since SM and cholesterol have a physical affinity and their concentrations in membranes commonly run in parallel (33), this may explain the lower microsomal UC levels in the two diet-fed groups.

**Fig. 6.** Rats were fed a standard low-fat diet (control) or a high-fat diet for 18 wk in total. After 10 wk, rats fed the high-fat diet were divided into the HF + CoQ, HF + olive oil, and HF subgroups. At the end of the experiment, the livers were excised, the microsomal fraction was isolated by ultracentrifugation, the lipids were extracted, and the concentrations of TG (A), unesterified cholesterol (UC; B), and cholesteryl ester (CE; C) were determined. D: TG-to-UC ratio. Data are means ± SE; n = 3 animals in the HF group, 5 animals in the control group, 6 animals in the HF + CoQ group, and 6 animals in the HF + olive oil group. ★P < 0.05 and ★★★P < 0.001 vs. the control group; ★P < 0.05 and ★★P < 0.01 vs. the HF + olive oil group; ★★P < 0.05 and ★★★P < 0.01 vs the HF group.

**Fig. 7.** The relationship between liver microsomal TG content and MTP activity after rats were fed the diets described in Figs. 3 and 6. \( r^2 = -0.57, P < 0.01 \).
MTP is required for both steps of VLDL synthesis, and its inhibition leads to more recycling of the TG produced by DGAT2 into the cytosol (20). Reduced MTP activity, therefore, might be expected to lead to decreased VLDL secretion.

In fact, however, the present data suggest that VLDL secretion was increased in the high-fat diet-fed rats, as usually occurs in NAFLD (Fig. 1) (2, 3, 44, 47). Our results show that feeding rats the high-fat diets significantly increases the levels of sphingomyelin (SM; A), phosphatidylcholine (PC; C), phosphatidyl ethanolamine (PE; D); phosphatidylserine (PS; E), and cardiolipin (Cln; F) were determined. G: PC-to-PE ratio; H: SM-to-UC ratio. Data are means ± SE; n = 3 animals in the HF group, 5 animals in the control group, 6 animals in the HF + CoQ group, and 6 animals in the HF + olive group. *P < 0.05 and **P < 0.01 vs. the control group; aP < 0.05 vs. the HF + olive oil group; bP < 0.05 vs. the HF group.

Fig. 8. Rats were fed a standard low-fat diet (control) or a high-fat diet for 18 wk in total. After 10 wk, rats fed the high-fat diet were divided into the HF + CoQ, HF + olive oil, and HF subgroups. At the end of the experiment, the livers were excised, the microsomal fraction was isolated by ultracentrifugation, the lipids were extracted, and the concentrations of sphingomyelin (SM; A), phosphatidylcholine (PC; C), phosphatidyl ethanolamine (PE; D); phosphatidylserine (PS; E), and cardiolipin (Cln; F) were determined. G: PC-to-PE ratio; H: SM-to-UC ratio. Data are means ± SE; n = 3 animals in the HF group, 5 animals in the control group, 6 animals in the HF + CoQ group, and 6 animals in the HF + olive group. *P < 0.05 and **P < 0.01 vs. the control group; aP < 0.05 vs. the HF + olive oil group; bP < 0.05 vs. the HF group.

In fact, however, the present data suggest that VLDL secretion was increased in the high-fat diet-fed rats, as usually occurs in NAFLD (Fig. 1) (2, 3, 44, 47). Our results show that feeding rats the high-fat diets significantly increases the levels of sphingomyelin (SM; A), phosphatidylcholine (PC; C), phosphatidyl ethanolamine (PE; D); phosphatidylserine (PS; E), and cardiolipin (Cln; F) were determined. G: PC-to-PE ratio; H: SM-to-UC ratio. Data are means ± SE; n = 3 animals in the HF group, 5 animals in the control group, 6 animals in the HF + CoQ group, and 6 animals in the HF + olive group. *P < 0.05 and **P < 0.01 vs. the control group; aP < 0.05 vs. the HF + olive oil group; bP < 0.05 vs. the HF group.
apoB48 in the liver (Fig. 2), whereas apoB mRNA concentrations remained unchanged in the absence of CoQ9 supplementation (Fig. 5), suggesting that apoB degradation is decreased in these animals and, thus, that the number of VLDL precursor particles formed is increased. High-fat diet feeding did not change the expression of mRNA for ARF1, which provides phosphatidic acid for TG synthesis (32), or DGAT1 but did cause an increase in the abundance of transcripts for DGAT2 (Fig. 5), which is specifically involved in TG synthesis for VLDL production in the ER membrane (18). Moreover, suppression of DGAT2, but not DGAT1, expression has been shown to protect against NAFLD in rats (12). The new evidence from our findings provides a possible explanation for the overproduction of VLDL in high-fat diet-induced NAFLD. The supply of TG for the TG-rich particle required in the second step of VLDL assembly is increased in the condition because there is a failure of insulin to promote recycling of TG to the cytosol (3, 18), and TG synthesis via DGAT2 may also be increased. According to Hussain et al. (23), this would lead to a rise in the number of nucleation sites for the formation of VLDL precursor particles, even though MTP activity is inhibited. Thus, a greater number of precursor particles would be formed, and the increased supply of TG for the TG-rich particles would increase their size. Since the size of the TG-rich particles determines the size of the VLDL secreted (18), this would lead to the overproduction of large VLDL in high-fat diet-induced NAFLD animals.

Oxidative stress resulting from fat accumulation in the liver in NAFLD is thought to be an important factor in the cellular damage that leads to NASH and other more serious liver diseases (1, 19). For this reason, it has been proposed that antioxidant therapy may be beneficial for patients with NAFLD or NASH (15, 28, 29). Of the naturally occurring lipid-soluble antioxidants, CoQ is the most effective in membranes (6). In addition to acting as an antioxidant, it increases membrane fluidity and permeability. Since lipid peroxidation causes damaging changes to membranes (34), it is possible that the concentration of CoQ is altered in NAFLD. CoQ concentrations in the rat liver have been shown to be modulated by dietary fat (21), and Petrosillo et al. (37) reported that the activity of liver mitochondrial NADH-CoQ oxidoreductase is decreased in rats with NAFLD induced by choline deficiency. In a recent study (40) using our high-fat diet-induced NAFLD rat model, we found that dietary CoQ9 had little effect on increasing liver apoB48 levels, inhibition of MTP and ACAT activity, and upregulation of the expression of mRNA for DGAT2, which controls the synthesis of TG for VLDL assembly, and that these changes, together with the lack of suppression of secretion by insulin, provide a possible explanation for the overproduction of VLDL associated with the disease. Our results also provide the novel information that dietary CoQ9 modulates VLDL processing after the development of hepatic steatosis so that apoB synthesis and microsomal TG content are increased and the ER membrane phospholipid content is altered. Although these modifications do not lead to further change VLDL levels in plasma, they may promote the secretion of larger, more atherogenic VLDL.

In summary, the findings reported here show, for the first time, that in high-fat diet-induced NAFLD there are disturbances in VLDL assembly in the ER of the liver caused by increased liver apoB48 levels, inhibition of MTP and ACAT activity, and upregulation of the expression of mRNA for DGAT2, which controls the synthesis of TG for VLDL assembly, and that these changes, together with the lack of suppression of secretion by insulin, provide a possible explanation for the overproduction of VLDL associated with the disease. Our results also provide the novel information that dietary CoQ9 modulates VLDL processing after the development of hepatic steatosis so that apoB synthesis and microsomal TG content are increased and the ER membrane phospholipid content is altered. Although these modifications do not lead to further change VLDL levels in plasma, they may promote the secretion of larger, more atherogenic particles.

ACKNOWLEDGMENTS

The authors thank Dr. Giorgio Borioni for supplying the CoQ9 monomethyl ether and Prof. Gian Paolo Littarru for the help with CoQ9 determinations. The authors are grateful to Dr. Maria José Martinez and Dr. Yolanda Chico for the help with the ACAT assays and to Antonio Di Virgilio and Agostino Eusepi for the skilled help with animal work.

GRANTS

G. Safwat was supported by a fellowship from the Embassy of the Arab Republic of Egypt and the Italian Health Ministry (ISS Ref. R26). A. Cano was a recipient of a predoctoral fellowship from the University of Basque Country. Work performed at the University of Basque Country was supported by the Basque government (PE07UN11 and IT325-07).

REFERENCES

VLDDL ASSEMBLY IN NONALCOHOLIC FATTY LIVER DISEASE


