Nonalcoholic steatohepatitis (NASH) progresses to liver fibrosis and cirrhosis, which can lead to life-threatening liver failure and the development of hepatocellular carcinoma. The aim of the present study was to create a rabbit model of NASH with advanced fibrosis (almost cirrhosis) by feeding the animals a diet supplemented with 0.75% cholesterol and 12% corn oil. After 9 months of feeding with this diet, the rabbits showed high total cholesterol levels in serum and liver tissues in the absence of insulin resistance. The livers became whitish and nodular. In addition, the number of rabbit macrophage antigen-positive cells and the expression of mRNAs for inflammatory cytokines showed a significant increase. Moreover, fibrotic septa composed of collagens and α-smooth muscle actin-positive cells were found between the central and portal veins, indicating alteration of the parenchymal architecture. There was also a marked increase of mRNAs for transforming growth factor-β1 and collagen 1A1. Comprehensive analysis of protein and gene expression revealed an imbalance of the antioxidant system and methionine metabolism. We also found that ezetimibe attenuated steatohepatitis in this model. In conclusion, the present rabbit model of NASH features advanced fibrosis that is close to cirrhosis and may be useful for analyzing the molecular mechanisms involved in the pathogenesis of human NASH. However, rabbits fed a HFD have a short lifespan attributable to heart failure accompanied by severe arteriosclerosis. This makes it difficult to study whether advanced fibrosis or even cirrhosis can be caused solely by HFD feeding. We therefore tried to improve the model and produce NASH with advanced fibrosis, which is more similar to the disease observed in humans that gradually develops after several decades. In the present study, this was accomplished by reducing the concentrations of cholesterol and corn oil in the diet and by prolonging the feeding period from 2 to 9 months.

A high-fat diet is one of the risk factors for metabolic syndrome, which is characterized by obesity, hyperlipidemia, hyperglycemia, and hypertension, and is frequently accompanied by life-threatening arteriosclerosis. A high-calorie, high-fat diet is also considered to cause nonalcoholic fatty liver disease (NAFLD), which covers a spectrum of disorders from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis. Recent clinical studies have shown that NAFLD is one of the common liver diseases that leads to cirrhosis and hepatocellular carcinoma in a manner similar to the clinical course of chronic viral hepatitis and alcohol abuse. However, the molecular mechanisms underlying the progression of NAFLD to an advanced stage with active inflammation and fibrosis are not fully understood. We recently reported a rabbit model of steatohepatitis that was generated by feeding the rabbit a high-fat and -cholesterol diet (HFD) supplemented with 20% corn oil and 1.25% (w/w) cholesterol for 8 weeks. The rabbit showed insulin resistance, accumulation of lipids in hepatocytes, activation of Kupffer cells (liver macrophages), mild fibrosis, and enhanced oxidative stress. Thus, we concluded that this model was useful for analyzing the molecular mechanisms involved in the pathogenesis of human NASH. However, rabbits fed a HFD have a short lifespan attributable to heart failure accompanied by severe arteriosclerosis. This makes it difficult to study whether advanced fibrosis or even cirrhosis can be caused solely by HFD feeding. We therefore tried to improve the model and produce NASH with advanced fibrosis, which is more similar to the disease observed in humans that gradually develops after several decades. In the present study, this was accomplished by reducing the concentrations of cholesterol and corn oil in the diet and by prolonging the feeding period from 2 to 9 months.
NAFLD/NASH is assumed to be most effectively improved by weight control and by restricting lipid and calorie intake, thereby leading to normalized lipid metabolism.9,10 Nevertheless, drug therapy would be a useful and an easy option because the modern lifestyle habits such as poor diet and lack of regular exercise are difficult to change. Ezetimibe is a relatively new and promising drug candidate for NAFLD/NASH therapy. Ezetimibe selectively inhibits cholesterol absorption via Niemann-Pick C1-like 1 (NPC1L1) protein in the brush border of the small intestine in humans, rodents, rabbits, and other species.11-15 It decreases the serum levels of low-density lipoprotein cholesterol and triglycerides (TGs) in humans16 and reduces plaque formation and improves lipids in a rabbit model of atherosclerosis.17 Recent reports have indicated that ezetimibe improves liver steatosis and insulin resistance in Zucker obese fatty rats18 and rats fed a methionine- and choline-deficient diet.19 Thus, ezetimibe is a potential new therapeutic agent for human NASH.20 In the present study, we also assessed the effect of ezetimibe on the development of NASH in our rabbit model.

Materials and Methods

Materials

Mouse monoclonal antibodies against α-smooth muscle actin (αSMA), rabbit macrophage (clone RAM-11), and 4-hydroxy-2-nonenal (4-HNE) were obtained from Sigma Chemical Co. (St. Louis, MO), Thermo Fisher Scientific (Fremont, CA), and Nikken Seil Co., Ltd. (Shizuoka, Japan), respectively. Enhanced chemiluminescence detection reagent was obtained from Amersham Scientific (Fremont, CA), and Nikken Seil Co., Ltd. (Shizuoka, Japan), respectively. 4-Methylumbelliferyl (MUB) and naphthaline-2-sulfonate (NSD) were purchased from Wako Pure Chemical Co. (Osaka, Japan). 4-hydroxy-2-nonenal (4-HNE) was obtained from Sigma Chemical Co. (OSaka, Japan).

Animals and Experimental Protocol

Pathogen-free male Japanese White rabbits, about 1-year-old and weighing 3.0 to 3.5 kg, were obtained from SLC (Shizuoka, Japan). As shown in Figure 1, we performed the following two experiments: i) rabbits were fasted 24 hours before sacrifice. The rabbits were anesthetized and laparotomized for blood and liver sampling. The portal vein was cannulated using an 18-gauge Teflon catheter. The liver of each animal was perfused with 100 ml of PBS to remove the blood. After harvest, the liver was cut into small pieces and fixed in 4% paraformaldehyde. Each sample was either frozen or embedded in paraffin. The experiments were conducted humanely in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Osaka City University School of Medicine.

Histochemical and Immunohistochemical Studies

Paraformaldehyde-fixed specimens were sectioned into 5-μm-thick sections and stained with H&E and 0.1% (w/v) Sirius red (Direct Red 80, Aldrich, Milwaukee, WI). Frozen sections (5-μm-thick) were stained with Oil red O (Wako Pure Chemical Co.). The areas stained by Sirius red and Oil red O were measured to assess the areas of connective tissue and lipid deposition, respectively, using an image analyzer (Lumina Vision, Mitani Corporation, Tokyo, Japan).

Immunohistochemical analysis was performed as described elsewhere.7 In brief, sections were deparaffinized, washed, and preincubated in 5% bovine serum albumin blocking solution, followed by overnight incubation at 4°C with antibodies against either αSMA at a dilution of 1:100, rabbit macrophage (RAM-11)21 at a dilution of 1:100, or 4-HNE at a concentration of 5 μg/ml.22 The sections were incubated with biotinylated sec-
secondary antibodies and reacted with horseradish peroxidase-conjugated streptavidin (Nichirei Biosciences Inc., Tokyo, Japan) and then treated with diaminobenzidine (DAKO, Glostrup, Denmark) for color development.

**Laboratory Tests**

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-Chol), TG, free fatty acid (FFA), and fasting glucose were measured at the Special Reference Laboratories (Osaka, Japan). Fractionation of serum cholesterol was performed by high-performance liquid chromatography at Skylight Biotech (Akita, Japan). The serum and urine levels of oxidative stress markers and antioxidants were measured at Nikken Seil Co., Ltd. Fasting serum insulin levels were measured using a rat insulin enzyme-linked immunosorbent assay kit and a rabbit insulin standard solution (Shibayagi Co. Ltd., Gunma, Japan). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula HOMA-IR = [fasting insulin (ng/ml) × 23.1] × fasting glucose (mg/dl) ÷ 405. Serum bile acid was measured using a Total Bile Acids Test Wako (Wako Pure Chemical Co.) according to the manufacturer’s instructions.

**Assay of Hepatic Total Cholesterol, Triglyceride, and Free Fatty Acid Levels**

Liver tissue (50 to 100 mg) was homogenized in 0.75 ml of methanol and chloroform (2:1), and lipids were extracted from the chloroform fraction. Then, the hepatic tissue levels of T-Chol and TG were determined using a Cholesterol E-Test Wako and Triglyceride E-Test Wako (Wako Pure Chemical Co.) according to the manufacturer’s instructions. The data were expressed as the amount of T-Chol (mg) or TG (mg)/liver wet weight (g).

For the hepatic FFA assay, liver tissue (10 mg) was homogenized in 0.2 ml of chloroform and 1% Triton X-100. Fatty acids were extracted in the chloroform fraction and air-dried to remove the chloroform. Then, the hepatic tissue FFA levels were determined using a Free Fatty Acid Quantification Kit (BioVision, Mountain View, CA) according to the manufacturer’s instructions. The data were expressed as the amount of FFA (nmol)/liver wet weight (mg).

**Quantitative Real-Time PCR**

Total RNA was extracted from the liver using Isogen (Nippon Gene Co. Ltd., Tokyo, Japan). cDNAs were synthesized with 1 µg of total RNA, ReverTra Ace (Toyobo, Osaka, Japan), and oligo(dT)12-18 primers according to the manufacturer’s instructions. Gene expression was measured by real-time PCR on an Applied Biosystems Prism 7500 system (Applied Biosystems, Foster City, CA) using cDNA, real-time PCR Master Mix Reagents (Toyobo), a set of gene-specific oligonucleotide primers, and the TaqMan probes listed in Table 1.

**Immunoblotting**

Protein samples (10 µg) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to Immobilon-P membranes. After blocking, the membranes were treated with the primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using the enhanced chemiluminescence system and documented with LAS 1000 (Fuji Photo Film, Kanagawa, Japan). The density of each band was analyzed using a GS-700 densitometer (Bio-Rad Laboratories, Hercules, CA).

**Assay of Hepatic 8-Hydroxy-2’-Deoxyguanosine Levels**

Liver tissue (100 to 200 mg) was homogenized in lysis buffer, and hepatic DNA was extracted using the DNA Extractor TIS kit (Wako Pure Chemical Co.). After the DNA was hydrolyzed, 8-hydroxy-2-deoxyguanosine levels in the liver were measured using a highly sensitive 8-hydroxy-2-deoxyguanosine enzyme-linked immunosorbent assay kit (Nikken Seil Co. Ltd.) according to the manufacturer’s instructions.

**Proteome Analysis**

Two-dimensional SDS-PAGE was performed by Towa Environment Science (Osaka, Japan). Proteins (100 µg) extracted from rabbit livers were applied to Immobiline DryStrips (pH 3 to 10). After isoelectric focusing, the proteins were separated by SDS-PAGE on 9 to 18 acrylamide gradient gels, visualized by SYPRO Ruby staining, scanned, and analyzed as described previously. Protein spots of interest were excised from the gels, digested in trypsin solution, dia lyzed, and then analyzed by electrospray ionization mass spectrometry. The proteins were identified from the obtained amino acid sequences using databases such as protein BLAST or FASTA.

**Microarray Analysis**

Total RNA was extracted from liver tissues with Isogen. Rabbit microarray chips were designed on the eArray system (Agilent Technologies, Palo Alto, CA) and were provided by Takara Bio Inc. (Shiga, Japan). The gene expression profile of HFD II-fed rabbits was compared with that of SD-fed rabbits. Genes showing differences in expression with an increase of more than fivefold or a decrease to <0.5-fold were recognized as up- or down-regulated genes, respectively, and were targeted for further analysis. The data are partially shown in Supplemental Table S2 (see http://ajp.amjpathol.org).

**Statistical Analysis**

Bar graphs present data as means ± SD of at least three independent experiments. Statistical analysis was per-
<table>
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<td>Col1A1</td>
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</table>

(Table continues)
Results

Hepatic Lipid Deposition in Rabbits Fed High-Fat and -Cholesterol Diet II

Rabbits fed HFD I for 2 months exhibited insulin resistance, hepatic steatosis, inflammation, oxidative stress, and mild fibrosis, thus, showing similarity to human NASH as we reported previously. After the feeding period was increased by reducing the fat and cholesterol content in the diet to 60% of the previous level (HFD II) to prolong rabbit survival, we were able to create a model with advanced hepatic fibrosis that was close to cirrhosis. As shown in Table 2, serum T-Chol and TG levels of rabbits fed HFD II for 9 months increased significantly compared with the levels in rabbits fed SD. The serum cholesterol was mainly very low-density lipoprotein and lipoprotein cholesterol according to the high-performance liquid chromatography analysis. However, serum AST and ALT levels in HFD II-fed rabbits did not change significantly compared with those in SD-fed rabbits. Fasting glucose and insulin values in HFD II-fed rabbits were lower than those in SD-fed rabbits, and HOMA-IR was reduced in HFD-fed rabbits.

The livers of HFD II-fed rabbits were enlarged and whitish with an irregular, partially nodular surface (Figure 2, A and B), showing an appearance that was totally different from the livers of SD-fed rabbits. H&E staining indicated degeneration of hepatocytes predominantly around the central vein area (Figure 2, C and D). At a higher magnification, H&E staining also revealed glassy degeneration of hepatocytes, which was similar to the ballooning of hepatocytes in human NASH, fibrosis, and bile duct proliferation (Figure 2, E and F), as well as atheroma in the aorta (Figure 2, G and H). The hepatocytes were strongly positive for Oil red O staining (Figure 3, A and B). Furthermore, hepatic T-Chol content in-
creased significantly in HFD II-fed rabbits (38.3 ± 21.5 μg/mg liver weight) compared with that in SD-fed rabbits (1.5 ± 0.2 μg/mg liver weight), although the hepatic TG and FFA contents were similar (Figure 3C), indicating that mainly cholesterol had accumulated in the hepatocytes of rabbits fed HFD II. Expression of genes related to fat metabolism, such as peroxisome proliferator-activated receptor-γ (PPARγ) and adipocyte lipid-binding protein (aP2), also known as fatty acid binding protein 4, increased

<table>
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<tr>
<th>Enzymes/lipids</th>
<th>SD</th>
<th>HFD II</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (IU/L)</td>
<td>43.4 ± 29.9</td>
<td>29.3 ± 22.4</td>
<td>NS</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>45.6 ± 36.6</td>
<td>19.7 ± 18.2</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>19.2 ± 3.8</td>
<td>1161.3 ± 406.5</td>
<td>P &lt; 0.01</td>
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<td>Chylomicron</td>
<td>0.2 ± 0.2</td>
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<td>Very low-density lipoprotein</td>
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</tr>
<tr>
<td>TG (mg/dl)</td>
<td>26.7 ± 11.9</td>
<td>205.5 ± 96.3</td>
<td>P &lt; 0.01</td>
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<tr>
<td>FFA (μEq/L)</td>
<td>279.7 ± 150.7</td>
<td>168.4 ± 107.9</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>132.7 ± 11.0</td>
<td>98.4 ± 47.3</td>
<td>P &lt; 0.05</td>
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<tr>
<td>Fasting insulin (ng/ml)</td>
<td>1.1 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>P &lt; 0.05</td>
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<tr>
<td>HOMA-IR</td>
<td>8.4 ± 2.3</td>
<td>5.2 ± 2.6</td>
<td>P &lt; 0.05</td>
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<tr>
<td>Bile acid</td>
<td>5.3 ± 1.3</td>
<td>33.7 ± 17.4</td>
<td>P &lt; 0.05</td>
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</table>

NS, not significant.

Table 2. Liver Enzymes and Lipid Profile in Serum

Figure 2. Steatosis and fibrosis in the liver of HFD II-fed rabbits. Rabbits were fed SD (A, C, and G) or HFD II (B, D, E, F, and H) for nine months. A and B: Macroscopic appearance of the livers of SD- and HFD II-fed rabbits. C–H: H&E staining of the liver (C–F) and aorta (G and H). Note that lipid-induced hepatic degeneration and liver fibrosis are predominantly seen in the livers (D–F) and that atheroma developed in the aorta (H) of HFD II-fed rabbits. Arrows in E, arrowheads in F, and asterisk in H indicate fibrotic septa, bile duct proliferation, and atheroma, respectively. P, portal vein; C, central vein. Scale bars = 100 μm.

Figure 3. Metabolism of lipid, glucose, and cholesterol in the livers of HFD II-fed rabbits. Rabbits were fed SD (A) or HFD II (B) for nine months. A and B: Oil red O staining. Note hepatic lipid deposits in HFD II-fed rabbits. Scale bars = 100 μm. C: Hepatic T-Chol (left), TG (middle), and FFA (right) contents. Note that the hepatic T-Chol content increased significantly in HFD II-fed rabbits. D: Expression of lipid, glucose, and cholesterol metabolism-related genes in the livers of SD-fed rabbits (white bars) and HFD II-fed rabbits (gray bars) analyzed by quantitative real-time PCR. *P < 0.05; **P < 0.01.

Figure 3C: Mainly cholesterol had accumulated in the hepatocytes of rabbits fed HFD II.
Hepatic Inflammation and Kupffer Cell Activation

RAM-11, an antibody directed against rabbit macrophages, recognizes activated Kupffer cells, the resident liver macrophages.21 In contrast to the livers of SD-fed rabbits (Figure 4A), in the livers of HFD II-fed rabbits, the number of RAM-11-positive (RAM-11+) cells increased drastically around the central vein area at sites where fatty degeneration of hepatocytes was evident (Figure 4B, see also Figure 2D). At a higher magnification (Figure 4B, inset), large RAM-11+ cells were localized in sinusoids, and they frequently contained vacuoles. In accordance with this observation, genes related to macrophage activation and cytokines, such as tumor necrosis factor α (TNFα); interleukin-1β (IL-1β), -10 (IL-10), and -18 (IL-18); Toll-like receptors 2 (TLR2) and 4 (TLR4); CD14 (a coreceptor with TLR 4); and MD2 (a complex with TLR4), were all induced in the livers of HFD II-fed rabbits (Figure 4C, Supplemental Table S2, see http://ajp.amjpathol.org).

Figure 4. Activation of macrophages in the livers of HFD II-fed rabbits. Rabbits were fed SD (A) or HFD II (B) for nine months. A and B: Immunohistochemistry for the rabbit macrophage RAM-11 clone. Note RAM-11+ cells around the central vein area in HFD II-fed rabbits, whereas these cells are absent in SD-fed rabbits. Inset: enlarged view of the box. Arrows indicate RAM-11+ cells with large vacuoles. Scale bars = 100 μm. P, portal vein; C, central vein. C: Expression of inflammatory genes in the livers of SD-fed rabbits (white bars) and HFD II-fed rabbits (gray bars) analyzed by quantitative real-time PCR. *P < 0.05, **P < 0.01.

A stress protein, heme oxygenase-1 (HO-1), was also increased significantly in the livers of HFD II-fed rabbits.

Advanced Hepatic Fibrosis in Rabbits Fed High-Fat and -Cholesterol Diet II

The livers of HFD II-fed rabbits showed evidence of advanced fibrosis. Sirius red staining showed a limited amount of red-colored collagen around portal and central veins in the control livers, whereas collagen deposition was marked in the livers of HFD II-fed rabbits (Figure 5, A and B). Fibrosis formed bridges between the central veins and central portal veins, indicating that the liver fibrosis was stage 3 (bridging fibrosis) or stage 4 (cirrhosis) according to the Brunt’s staging score. The fibrotic septa were composed of cells positive for αSMA, a marker of activated stellate cells and myofibroblasts (Figure 5, C and D). Upregulated expression of αSMA in HFD II-fed rabbits was confirmed by immunoblotting (Figure 5E). In addition, expression of genes associated with fibrosis, such as transforming growth factor β1 (TGFβ1), collagens 1A1 (Col1A1) and 3A1 (Col3A1), matrix metalloproteinases-2 (MMP-2) and -9 (MMP-9), and tissue inhibitors of metalloproteinases-1 (TIMP-1) and -2 (TIMP-2), increased in the livers of HFD II-fed rabbits (Figure 5F, Supplemental Table S2, see http://ajp.amjpathol.org). Furthermore, genes for other matrix proteins such as...
collagen 8A1, MMP-12, TIMP-3, lumican, decorin, and biglycan showed increased expression in the livers of HFD II-fed rabbits (Supplemental Table S2, see http://ajp.amjpathol.org).

**Oxidative Stress and Antioxidant Imbalance in Rabbits Fed High-Fat and -Cholesterol Diet II**

Immunohistochemical analysis showed that 4-HNE adduct formation was rare in SD-fed rabbits but was prominent in HFD II-fed rabbits. The 4-HNE adducts were present in the cytoplasm of sinusoidal cells and hepatocytes around the central vein areas (Figure 6, A–C), indicating increased lipid peroxidation in HFD II-fed rabbit livers as in human NASH. Moreover, the 8-hydroxy-2′-deoxyguanosine level increased, although not significantly, in both liver tissue and urine of HFD II-fed rabbits (Figure 6D, Table 3). As a measure of the presence of oxidative stress, the hepatic level of glutathione was determined in SD- and HFD II-fed rabbits; HFD II significantly reduced its levels (Figure 6E). In addition, the expression of genes for glutathione S-transferase (GST) and other antioxidant molecules, such as Cu,Zn-superoxide dismutase (Cu,Zn-SOD) and Mn-superoxide dismutase (Mn-SOD), decreased significantly in HFD II-fed rabbits (Figure 6F, Supplemental Table S2, see http://ajp.amjpathol.org). We measured the serum or urine levels of molecules that reflect oxidative stress. The levels of copper, δ-tocopherol, and coenzyme Q10 (ubiquinol and ubiquinone) increased significantly in HFD II-fed rabbits, whereas folic acid and vitamin A decreased significantly (Table 3). Comparison of hepatic protein distribution profiles on two-dimensional SDS-PAGE gels between SD- and HFD II-fed rabbits revealed proteins that were both up- and down-regulated by HFD II (Figure 7). GST was identified as a markedly down-regulated protein in HFD II-fed rabbits. Other down-regulated proteins in HFD II-fed rabbits by proteome analysis were glycine N-methyltransferase (GNMT), methionine adenosyltransferase 1 (MAT1), and sulfotransferase 3A1 (ST3A1). These genes also showed significantly lower expression in the livers of HFD II-fed rabbits (Figure 6F).

### Table 3. Profile of Oxidative Stress Markers and Antioxidants in Serum and Urine

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<tr>
<td>8-OHdG (ng/mg creatinine)</td>
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<td>Isoprostane (ng/mg creatinine)</td>
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<td>Lipid peroxide (nmol/ml)</td>
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<td>3.7 ± 0.4</td>
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<td><strong>Antioxidants</strong></td>
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<td>Iron (μg/dl)</td>
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<td>Copper (μg/dl)</td>
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<td>Folic acid (ng/ml)</td>
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<tr>
<td>α-Tocopherol (μg/dl)</td>
<td>95.0 ± 81.4</td>
<td>3499.7 ± 5040.3</td>
<td>Serum</td>
<td>NS</td>
</tr>
<tr>
<td>β-Tocopherol (μg/dl)</td>
<td>&lt;2.5</td>
<td>31.2 ± 10.4</td>
<td>Serum</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>γ-Tocopherol (μg/dl)</td>
<td>6.5 ± 2.7</td>
<td>706.9 ± 944.2</td>
<td>Serum</td>
<td>NS</td>
</tr>
<tr>
<td>Coenzyme Q10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ubiquinol (nmol/L)</td>
<td>58.0 ± 10.8</td>
<td>489.3 ± 151.3</td>
<td>Serum</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Ubiquinone (nmol/L)</td>
<td>N.D.</td>
<td>36.7 ± 23.7</td>
<td>Serum</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

NS, not significant.

**Effect of Ezetimibe on the Rabbit NASH Model**

To further evaluate the rabbit NASH model, we investigated the effect of a known compound that suppresses the occurrence of NASH through a known mechanism.
versus 27.4 sion of genes associated with liver fibrosis, such as Moreover, ezetimibe significantly decreased the expres-
pressed by the ezetimibe treatment (Figure 9, A–D). T-Cho and TG (Figure 8G). Furthermore, liver fibrosis,
(mildly induced in this 2-month HFD I model, was sup-
plementation showed that ezetimibe suppressed fat deposition
untreated rabbits during the course of ezetimibe-treat-
lower in HFD I-fed rabbits treated with ezetimibe than in
rabbits showed normal increases in body weight (data
not shown). Levels of AST, ALT, and TG remained
within the normal range in rabbits fed HFD I with or without
ezetimibe (AST, 38.0 ± 18.4 versus 20.8 ± 7.4 IU/L; ALT,
11.0 ± 1.0 versus 7.8 ± 3.0 IU/L; and TG, 31.0 ± 9.5
versus 27.4 ± 16.8 mg/dl). Serum T-Cho was significantly
lower in HFD I-fed rabbits treated with ezetimibe than in
untreated rabbits during the course of ezetimibe-treat-
ment for up to 8 weeks (Figure 8A). A histological exam-
ination showed that ezetimibe suppressed fat deposition
(Figure 8, B–F) and reduced the hepatic content of total
T-Cho and TG (Figure 8G). Furthermore, liver fibrosis,
mildly induced in this 2-month HFD I model, was sup-
pressed by the ezetimibe treatment (Figure 9, A–D).
Moreover, ezetimibe significantly decreased the expres-
sion of genes associated with liver fibrosis, such as
TGFβ1, MMP-9, and TIMP-1 and -2. The drug also sup-
pressed expression of Col1A1 and MMP-2, although
the difference was not significant (Figure 9E).

Discussion
There are several important differences in characteristics of NASH between this rabbit model and humans. Hepatic levels of cholesterol, but not of TG and FFA, exhibited a marked increase in HFD II-fed rabbits compared with those in SD-fed rabbits, and the PPARγ and aP2 mRNA levels were induced in the HFD II model in a manner similar to that observed in human NASH (Figure 3, C and D). NAFLD shows various patterns of lipid deposition in the liver, which may be influenced by the diet composition. In this context, increased PPARγ and aP2 may contribute to a decrease in cholesterol level in hepatocytes of this rabbit model induced by high-cholesterol diets. Mitochondrial free cholesterol, but not TG and FFA, sensitizes hepatocytes to TNFα- and Fas-induced apoptosis through mitochondrial glutathione exhaustion. We also observed a marked reduction in glutathione and glutathione-metabolic enzymes in the HFD II-fed rabbit livers (Figure 6, E and F). In this context, it is likely that cholesterol overload, together with a dysregulated antioxidative system in hepatocytes, may trigger liver injury. Furthermore, this rabbit model showed increased levels

Because a marked increase in serum and hepatic T-Cho
levels was evident in our HFD-fed rabbits, we tested
ezetimibe, a relatively new compound that inhibits
NPC1L1 in hepatocytes and the intestine. Rabbits were
fed HFD I (the original diet) with or without ezetimibe (0.6
mg/kg/day) (HFD I or HFD I + Ez) for two months. Histological sections
were prepared from HFD I-fed (B and D) and HFD I + Ez-fed rabbits (C and
red O staining. Scale bars = 100 μM. F1: Quantification of the lipid droplet
area in the liver. Oil red O-stained areas were measured in the livers of HFD
I and HFD I + Ez-fed rabbits. The stained area was significantly smaller in
HFD I + Ez-fed rabbits. G: Hepatic total cholesterol and triglyceride contents.
P, portal vein; C, central vein. HFD I, n = 5. HFD I + Ez, n = 5. *P < 0.05,
**P < 0.01.
load and the resulting hepatocyte dysfunction in cirrhosis were reduced by ezetimibe administration. Scale bars /H11001

8. Histological sections were prepared from HFD I-fed (A)

NASH patients,32 serum and hepatic levels of FFA did not increase and adiponectin decreases in the serum of protein 2 (data not shown).

export pump and multidrug resistance-associated protein 2 (data not shown).

Although it has been reported that FFA and leptin increase and adiponectin decreases in the serum of NASH patients,34 serum and hepatic levels of FFA did not change in this rabbit model, possibly owing to the lack of obesity. A recent study reported the reduction of serum adiponectin in a rabbit model fed 10% lard and 2% cholesterol-containing HFD for 8 and 12 weeks.33 Unfortunately, we were unable to determine the adiponectin and leptin levels in our rabbit model for unknown reasons (data not shown).

We also observed no increase in fasting glucose, fasting insulin, or HOMA-IR in this rabbit model. However, as stated above, this rabbit model showed no obesity and failed to induce high FFA levels in the liver and serum, which may be reasons for its failure to induce type II diabetes and insulin resistance. Furthermore, as shown in Figure 3D, G6Pase and PEPCK mRNA expression levels were markedly suppressed in the HFD II model, similar to a report on cirrhotic NASH patients.34 Cholesterol overload and the resulting hepatocyte dysfunction in cirrhosis are assumed to be the reasons for this down-regulation, indicating the actual impairment of glucose metabolism in the HFD II liver.

The serum ALT level has long been used as a surrogate marker for liver injury. However, ALT values do not correlate well with the severity of liver injury in human NAFLD.35,36 Similar to that in patients with NAFLD but that inuniluke viral or drug-induced hepatitis, the ALT level in this rabbit model remained unchanged, but the mechanism remains unknown and should be clarified in future research.

Gut-derived lipopolysaccharide activates Kupffer cells by activating lipopolysaccharide receptors,37 leading to increased production of inflammatory cytokines such as TNFα, IL-6, and IL-8.38,39 The development of steatohepatitis in a NASH model, induced by a methionine- and choline-deficient diet, was partly inhibited in TLR4 mutant mice,40,41 suggesting a role for TLR4-dependent signaling in the occurrence of this type of liver damage. In addition, the lipoprotein component of endotoxin from Gram-negative and -positive bacteria activates TLR2 and/or TLR4, which leads to common downstream activation of TRAF6 via the adapter molecule MyD88.42 This cascade of events culminates in nuclear factor-κB activation, leading to the induction of TNFα and other proinflammatory cytokines.42 Although TNFα has been identified as a central mediator contributing to insulin resistance and liver damage in NASH, little is known about the role of TLR2 or TLR4 in the induction of TNFα. In the present study, we observed an increase in the expression of TLR4/CD14/MD2 and TLR2 and cytokine induction in HFD II-fed rabbits. Persistent hepatic inflammation also triggers the activation of stellate cells and excess collagen production, resulting in the development of liver fibrosis.43,44 Stellate cells are activated by LPS through TLR4/CD14/MD2 signaling.45,46 Thus, the role of LPS in triggering steatohepatitis in this HFD II model deserves to be studied further in relation to hepatic fibrogenesis.

In the present study, we showed a reduction in hepatic glutathione content and decreased GST and SOD mRNA expression. Serum levels of antioxidants such as vitamin E, copper, and coenzyme Q10 increased significantly, but the levels of vitamin A and folic acid decreased significantly. As a result, there was an imbalance between oxidative stress and antioxidant protection systems in the present steatohepatitis model, as in human NASH patients.47–52 The molecular mechanisms leading to the dysregulation of small antioxidant molecules are currently unknown. Most vitamin A in the body (approximately 70%) is usually stored in quiescent hepatic stellate cells,53 but vitamin A storage is impeded when stellate cells are activated, under which activation they express αSMA and produce extracellular matrix materials including type I collagen.43,44 Thus, the reduction of serum vitamin A levels may reflect activation of stellate cells and the progression of fibrosis in our rabbits with HFD II-induced NASH. Folic acid is reportedly involved in the maintenance of normal concentrations of homocysteine, methionine, and S-adenosylmethionine.54 Folic acid deficiency and abnormal hepatic methionine metabolism
are characteristics of alcoholic liver disease. Therefore, the reduction in serum folic acid observed in our rabbit NASH model might be a common feature of both alcoholic and nonalcoholic liver disease. Furthermore, the proteome analysis identified a reduction in GNMT and MAT1, which are associated with liver steatosis and fibrosis, in HFD II-fed rabbits. 

Martinez-Chantar et al. showed that GNMT-knockout mice exhibited an elevation in serum aminotransferase, methionine, and S-adenosylmethionine and developed hepatic steatosis, fibrosis, and hepatocellular carcinoma. MAT1A-knockout mice were also reported to show liver steatosis. These enzymes play an important role in the synthesis and degradation of S-adenosylmethionine. Thus, an imbalance in S-adenosylmethionine and methionine metabolism may play a role in the development of steatohepatitis in our model.

Phagocytic NADPH oxidases, such as gp91-phox, p40-phox, p67-phox, and p22-phox, increased in the present rabbit model (Supplemental Table S2, see http://ajp.amjpathol.org); however, the role of NADPH oxidase, a key molecule in the development of atherosclerosis, in NASH is poorly understood. In alcoholic liver injury, NADPH oxidase is important for reactive oxygen species production in Kupffer cells and in hepatic stellate cells that initiate and promote liver injury. We previously reported that the phagocytic activity of Kupffer cells promotes oxidative stress, inflammation, and fibrosis in steatohepatitis. In this context, NADPH oxidase could play a prominent role in the pathogenesis of human NASH.

In the present study, we created a rabbit model of steatohepatitis, in which advanced fibrosis (close to cirrhosis) was produced by feeding a HFD. Hypercholesterolemia is a risk factor for liver injury as well as for atherosclerosis; therefore, lowering the serum T-Chol level by dieting or by medications that reduce the synthesis and absorption of cholesterol could be a promising therapy for NAFLD including NASH. In this context, statins, which are HMG-CoA reductase inhibitors, have been reported to improve NASH. Rallidis et al. showed that pravastatin treatment lowered serum ALT and improved histological steatosis in 5 NASH patients. Hyogo et al. treated patients with atorvastatin and found that 23 patients (74.2%) exhibited normalized transaminases and histological improvement of liver steatosis and the NAFLD activity score, and these changes were accompanied by a significant increase in serum adiponectin and a significant decrease in serum TNFα. In the present study, we studied the effect of ezetimibe, which inhibits NPC1L1 and therefore blocks intestinal absorption of cholesterol from the diet or that excreted in the bile. The effect of ezetimibe on NASH has already been reported in rat models. Assy et al. studied methionine and choline deficiency-induced steatohepatitis in rats, in which ezetimibe administration alone or together with rosiglitazone, metformin, and valsartan reduced the hepatic levels of TG, T-Chol, and malondialdehyde and also significantly attenuated histological steatosis. In Zucker obese fatty rats, Deushi et al. reported that ezetimibe administration reduced the serum and hepatic levels of T-Chol and TG, the number of Oil red O-positive hepatocytes, Sirius red-stained collagen deposition, and αSMA expression. Supporting these observations, we demonstrated the usefulness of ezetimibe treatment in a rabbit NASH model. Because human lipid metabolism is similar to that of rabbits but is different from that of mice and rats, our results strengthen the potential of ezetimibe for controlling fat deposition and fibrosis in human NASH. Interestingly, ezetimibe not only improved HFD I-induced liver steatosis but also reduced fibrosis and the number of αSMA+ cells. In chronic liver disease, TGFβ1 plays an important role in the progression of liver fibrosis and stellate cell activation. TGFβ1 produced by Kupffer cells and stellate cells activates stellate cells in a paracrine as well as an autocrine manner and stimulates type I collagen production. In our rabbit model, ezetimibe reduced TGFβ1 and type I collagen expression in the liver. Unlike mice, humans and rabbits show abundant NPC1L1 expression in the liver. Further studies are required to clarify the effect of ezetimibe prophylactically after the HFD-fed rabbits have already developed steatohepatitis and to examine whether ezetimibe directly affects cholesterol metabolism in hepatocytes, thereby participating in the local prevention of fibrosis caused by abnormal cholesterol metabolism.

In conclusion, rabbits fed HFD II for 9 months developed steatohepatitis with advanced fibrosis accompanied by the augmented expression of relevant genes. We demonstrated the presence of an imbalance between oxidative stress and antioxidant levels in HFD II-fed rabbits. Ezetimibe therapy was promising for alleviating the pathological changes in this model, suggesting the potential usefulness of this compound for human liver diseases caused by cholesterol overload.

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References


