A growing body of evidence shows that lipid metabolism in the liver plays a crucial role in the pathogenesis of metabolic syndrome, which is associated with manifestation of obesity, dyslipidemia, type 2 diabetes mellitus, atherosclerosis, and non-alcoholic fatty liver disease (NAFLD)1-3). To study the pathogenesis of metabolic syndrome, many diet-induced animal models using mice and rats are available. In contrast to human lipid metabolism, however, mice are high-density lipoprotein cholesterol-dominant in their serum lipid profile and are essentially resistant to a high-fat/high-cholesterol diet (HcD) to develop atherosclerosis. This is explained by complete absence of expression of several genes that are critical for lipid metabolism and atherogenesis1-5). There are some mice models of

**Introduction**

A growing body of evidence shows that lipid metabolism in the liver plays a crucial role in the pathogenesis of metabolic syndrome, which is associated with manifestation of obesity, dyslipidemia, type 2 diabetes mellitus, atherosclerosis, and non-alcoholic fatty liver disease (NAFLD)1-3). To study the pathogenesis of metabolic syndrome, many diet-induced animal models using mice and rats are available. In contrast to human lipid metabolism, however, mice are high-density lipoprotein cholesterol-dominant in their serum lipid profile and are essentially resistant to a high-fat/high-cholesterol diet (HcD) to develop atherosclerosis. This is explained by complete absence of expression of several genes that are critical for lipid metabolism and atherogenesis1-5). There are some mice models of...
NAFLD, which range from hepatic isolated steatosis to non-alcoholic steatohepatitis (NASH), however, they do not genuinely reflect the pathology of human NASH\textsuperscript{1, 9}. For example, mice that are fed a methionine and choline-deficient diet manifest NASH lesions with severe body weight loss and hypolipidemia and do not recapitulate the full spectrum of metabolic syndrome\textsuperscript{6-8}. The pathogenesis underlying the transition to NASH and subsequent end-stage liver cirrhosis and hepatocellular carcinoma, remains to be elucidated\textsuperscript{2, 3}.

In contrast, recent studies, including our own, suggest that bile acid (BA), as well as cholesterol metabolism, are involved in the pathogenesis of NAFLD\textsuperscript{9-11}. BAs are known to contain variable derivatives of cholic acid (CA), and each BA displays a specific affinity to the BA receptors and functions as a signaling molecule with distinctive effects to induce oxidative stress, cytotoxicity and apoptosis\textsuperscript{12, 13}. Metabolic syndrome has actually been reported to be related with higher levels of secondary BAs, such as deoxy-CA\textsuperscript{13}. In this scenario, efficient hepatic and intestinal excretion, absorption and transport of lipids and BAs, via enterohepatic and systemic circulation, would be expected to have diverse and key roles in the process of nutrient metabolism and also in the initiation to the progression of metabolic syndrome\textsuperscript{5, 9-11}.

Our laboratory has recently established a novel animal model for studies of hyperlipidemia-induced atherosclerosis using the world’s smallest Microminipigs\textsuperscript{TM} (µMPs), in which CA supplementation is not necessary to induce atherosclerosis\textsuperscript{14, 15}. Unlike popular mouse and rabbit models, swine represent a potentially more appropriate experimental model, since their lipoprotein metabolism, as well as their anatomy, physiology and feeding and sleep habits, are much similar to those of humans\textsuperscript{15, 16}.

**Aim**

Our specific aim was to re-evaluate the effects of CA on obesity, serum lipid metabolism, atherosclerosis and hepatic injuries, and also to evaluate those on hepatic and systemic macrophage activation, which would accelerate atherogenesis and hepatic injuries in HcD-fed µMPs.

**Methods**

**Animals and Diets**

Three-month-old male µMPs weighing 5 – 6 kg were used for all experiments. The µMPs were maintained in a room temperature of 24°C ± 3°C at 50% ± 20% relative humidity in a light-controlled facility (on a 12 –h light/dark cycle) with ad libitum access to tap water. Body weight (BW) was measured once a week in a fasted state. The swine were fed a normal chow diet (NcD; Kodakara 73, Marubeni Nisshin Feed, Tokyo, Japan) or special diets on a daily basis (3% of BW/day). The HcD consisted of NcD supplemented with 12% lard (Miyoshi Oil & Fat, Tokyo Japan) and 1.5% cholesterol (Wako Pure Chemical, Osaka, Japan). HcD with cholic acid (CA; Wako Pure Chemical) diet (HcD + CA) contained 0.7% CA in HcD. Fifteen µMPs were divided into three groups (n = 5 in each); NcD group (one µMP died within the experimental period), HcD group, and HcD + CA group. The blood glucose, blood cell counts, blood chemistry, lipoprotein levels and blood pressure were measured every two weeks in a fasted state. All µMPs were anesthetized and sacrificed via bilateral axillary artery exsanguination, and all of organs were excised, cut into appropriately sized pieces, and used for analyses after measuring the weight of each organ. We collected serum samples from the axillary vein and artery at sacrifice.

**Ethical Considerations**

All the protocols were approved by Ethics Committee of Animal Care and Experimentation, Kagoshima University, and were performed according to the Institutional Guidelines for Animal Experiments and the Law (no. 105) and Notification (no. 6) of the Japanese Government. The present study was also performed in accordance with animal welfare bylaws of Shin Nippon Biochemical Laboratories Ltd. (Kagoshima, Japan), a facility fully accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and approved by the International Animal Care and Use Committee.

**Analyses of Hepatic, Fecal and Serum Lipid and Bile Acid Contents**

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), and alkaline phosphatase (ALP), were measured using an assay kit (Wako Pure Chemical). To examine lipid and bile acid (BA) contents in the liver, feces and serum, each frozen sample was homogenized and extracted with chloroform-methanol (2/1 v/v) and ethanol solution, as previously described\textsuperscript{7, 17}. The organic phase was dried and reconstituted in 2-propanol. Then, triglyceride (TG), free fatty acid (FFA), cholesteryl ester (CE), and BA contents were determined using an assay kit (Wako Pure Chemical).

**Computed Tomography**

Abdominal computed tomography (CT) images were obtained before the experiments and after eight-week feeding. The fat thickness of the back subcutane-
ous adipose tissue was measured and percent increase of the fat thickness was calculated.

**Serum Lipoprotein Analyses**

After the eight-week feeding, µMPs were fasted for 20 h, and blood was collected from the axillary artery into a tube containing five µl of 0.5 mol/L ethylenediaminetetraacetic acid. The samples were centrifuged for 12 min at 5,500 g at 4°C and the serum was stored at −80°C until use. Total cholesterol (T-cho), chylomicron (CM), very low-density lipoprotein- (VLDL-cho), low-density lipoprotein- (LDL-cho), and high-density lipoprotein-cholesterol (HDL-cho), and TG were analyzed by an automated agarose gel electrophoresis apparatus (Epalyzer 2, Helena Laboratories, Saitama, Japan).

**Serum Cytokine Analysis**

Serum collected at eight-week feeding was assayed for interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) using porcine-specific ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Blood Pressure**

The arterial blood pressures were measured at the foreleg in conscious µMPs using a Manchette method with an apparatus made for human pediatric use.

**Histology and Immunohistochemistry**

The resected organs were fixed with 10% neutral buffered formalin for 24 h, and serial paraffin sections were applied to hematoxylin and eosin (H&E) stain or immunohistochemistry (IHC). Images from H&E, oil-red O, combined elastic and Masson trichrome (E-M), IHC and immunofluorescence (IF) stains were captured and quantified using a NIS-Elements D software program (Epalyzer 2, Helena Laboratories, Saitama, Japan)18). The degree of lipid accumulation (steatosis score) in H&E- and oil-red O-stained frozen tissue sections was categorized into four grades as follows: no lipid droplets (score 0); lipid droplets in <33% of hepatocytes (score 1); lipid droplets 33%–66% of hepatocytes (score 2) and lipid droplets in >66% of hepatocytes (score 3)6, 8).

To evaluate accumulation of macrophages in each tissue, IHC using a polyclonal rabbit anti-human lysozyme antibody were performed (1:1,000; Dako, Glostrup, Denmark)15). The number of lysozyme-positive macrophages was counted in 10 randomly selected fields in each sections. To assess activation of stellate cells, the liver tissues were stained with a monoclonal mouse anti-human smooth muscle actin (α-SMA; Clone 1A4) antibody (1:100; Dako), and positive stellate cells was counted in 10 randomly selected fields per section as previously described7).

Hepatic lymphocytic infiltration (inflammation score) was classified into four grades as following: no inflammation (score 0); <10 inflammatory foci, each consisting of >5 inflammatory cells (score 1); ≥10 inflammatory foci (score 2) or uncountable diffuse or fused inflammatory foci (score 3)6, 8). The degree of liver cell ballooning (ballooning score) was classified into three grades as follows: none (score 0); few balloon cells (score 1) or many balloon cells/prominent ballooning (score 2). Hepatic fibrosis was quantified by combined E-M stain. To determine expression of reactive oxygen species (ROS) and oxidative stress in the liver, we used an anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) monoclonal antibody (1:100; Japan Institute for the Control of Aging, Fukui, Japan) and anti-4-hydroxy-2-nonenal (4-HNE) monoclonal antibody (1:100; Japan Institute for the Control of Aging) for IHC and quantified the number of cells positive for either antibody in five randomly selected fields per section as previously described6, 7). The endogenous peroxidase activity was not blocked in the IHC for oxidative stress markers.

To evaluate the islet change and visceral adiposity, the pancreatic islet area and the size of visceral adipocytes were measured in five randomly selected fields per section using NIS-Elements D software program.

All histological slides were evaluated by two independent observers (S.Y. and H.K.), who were blinded to all data. In the case of disagreement, a consensus score was determined by a third observer (A.T.).

**Assessment of Atherosclerosis**

Each aorta was cut open longitudinally and fixed with 10% neutral buffered formalin for 24 h, then the aorta was stained with oil-red O stain for 20 min at room temperature. The en face images of the aorta were captured with a digital camera. The oil-red O-positive area relative to the whole surface area was measured with NIH image software program19). For histological examinations, six-step sections of every 1 cm of abdominal aorta were stained with H&E and IHC. For quantitative analysis, images of the six-step sections were scanned using NIS-Elements D software program to evaluate thickened intimal area20).

**Hepatic Apoptosis**

To determine apoptotic cell counts in the liver, we conducted TUNEL assays using an In Situ Cell Death Detection Kit (Roche Applied Science, Lewes, UK). The formalin-fixed liver sections were labeled with TUNEL reaction mixture, incubated with DAPI (1 µg/mL; Abbott Molecular Inc., Des Plaines, IL, USA), and visualized with anti-fluorescein-antibodies.
Serum thiobarbituric acid reactive substances (TBARS) levels were measured using a TBARS Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). The results are expressed as nM malondialdehyde (MDA)/mg LDL protein.

Monitoring of Intracellular O$_2^-$ Levels by Dihydroethidium Stain

In situ O$_2^-$ staining was performed using frozen liver sections to determine the levels of ROS and oxidative stress in hepatocytes. Frozen sections (3 µm in thickness) were stained with dihydroethidium (DHE;...
5 μM) fluorescent dye (Molecular Probes, Eugene, OR, USA) for in situ O₂⁻ imaging. The oxidative fluorescent dye was freely permeable to cells, and in the presence of O₂⁻ was oxidized to ethidium bromide (EtBr), which was trapped by intercalation with DNA. After staining, we quantified the number of EtBr-positive nuclei in 10 randomly selected fields per section under BX51N-34 fluorescence microscope.

**Table 1.** Serum lipid profile and BA level in µMPs

| Serum        | NcD (n=4)      | HcD (n=5)      | HcD + CA (n=5) |
|--------------|----------------|----------------|----------------|
| T-cho (mg/dL)| 73.5 ± 6.1     | 609.0 ± 48.4***| 744.8 ± 121.2**|
| CM (mg/dL)   | 1.5 ± 0.6      | 19.4 ± 2.7**   | 28.2 ± 9.2*    |
| VLDL-cho (mg/dL) | 4.5 ± 0.6   | 33.2 ± 7.3***  | 28.4 ± 7.0*    |
| LDL-cho (mg/dL) | 37.0 ± 7.0  | 408.5 ± 29.2***| 513.3 ± 93.3**|
| HDL-cho (mg/dL) | 30.3 ± 1.4  | 152.0 ± 10.3***| 182.0 ± 20.9***|
| TG (mg/dL)   | 45.3 ± 8.6     | 39.0 ± 5.8     | 70.5 ± 16.7#   |
| %LDL (%)     | 49.3 ± 5.0     | 68.0 ± 1.4*    | 68.0 ± 2.3**   |
| %HDL (%)     | 42.0 ± 3.5     | 24.0 ± 1.3***  | 23.3 ± 2.6**   |
| BA (µM)      | 0.4 ± 0.10     | 0.8 ± 0.04**   | 1.1 ± 0.38*    |

Values are means ± SE. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. NcD-fed µMPs; and #P < 0.05 vs. HcD-fed µMPs.

**Statistical Analyses**

All values are expressed as mean ± SE. Significant differences were analyzed using Student’s t-test, Welch’s t-test or one-way ANOVA. In all cases in which the ANOVA method was used to analyze non-parametric data, Tukey’s multiple comparison post hoc test was used. p-values of < 0.05 were considered to indicate statistical significance.

**Results**

**Body Weight and Visceral Adiposity**

The BW of µMPs in each group increased gradually. HcD and HcD + CA groups displayed moderate obesity in comparison to NcD group, however, no statistical difference was observed among three groups after eight weeks (Fig. 1A). The relative organ weights of omentum, mesenterium, and liver, but not the spleen, of HcD + CA-fed µMPs increased in comparison to NcD- and HcD-fed µMPs (Fig. 1B). Histologically, the diameter of adipocytes of white adipose tissue (WAT) in HcD + CA-fed µMPs was significantly larger than that in NcD- and HcD-fed µMPs (Fig. 1C). The food consumption similarly increased in three groups along with the increase in BW (data not shown).

**Subcutaneous Fat Thickness in Abdominal CT**

The fat thickness of the back (% increase) before and after eight-week feeding was significantly increased in HcD- and HcD + CA-fed µMPs in comparison to NcD-fed µMPs (NcD: 148% ± 39% vs. HcD: 235% ± 22% and HcD + CA: 232% ± 12%, P = 0.039 and P = 0.027, respectively), but there was no significant difference between HcD- and HcD + CA-fed µMPs.
ence in the serum lipid profiles between HcD-fed and HcD + CA-fed μMPs (Table 1).

### Serum Inflammatory Cytokine Profile

After eight-week feeding, the serum levels of
fed µMPs at six weeks (data not shown). The pancreatic tissues from three groups showed no remarkable change, and their islet areas were not significantly different (data not shown). The blood pressure did not significantly increase in any groups (systolic: 85–95 mmHg and diastolic: 45–60 mmHg).

**Blood Glucose and Blood Pressure**

The blood glucose levels did not increase in any groups after eight weeks, and no significant differences were found among three groups, with exception of higher blood glucose in HcD+CA-fed µMPs in comparison to HcD−fed µMPs at two weeks and NcD-fed µMPs at six weeks (data not shown). The blood pressure did not significantly increase in any groups (systolic: 85–95 mmHg and diastolic: 45–60 mmHg).

**Progressed Aortic Atherosclerosis in HcD + CA-fed µMPs**

The *en face* analysis of aortas showed that atherosclerosis was pronounced in HcD + CA-fed µMPs in comparison to NcD− and HcD-fed µMPs (**Fig. 2A**), even though the serum lipid levels was not different in...
HcD- and HcD + CA- fed groups. Thickness of the intima of aortas was significantly increased in HcD- and HcD + CA-fed µMPs than that in NcD-fed µMPs. The thickened intima consisted of accumulation of macrophages and significantly larger numbers of macrophages were infiltrating the upper layer of aortic media in HcD + CA-fed µMPs (Fig. 2B).

**Serum Bile Acid and Fecal Lipid Profile and Bile Acid**

The serum bile acid (BA) levels in HcD- and HcD + CA-fed µMPs were significantly higher than those in NcD-fed µMPs (Table 1). Both HcD- and HcD + CA-fed µMPs showed increased levels of fecal CE, FFA and TG in comparison to NcD-fed µMPs. On the other hand, the fecal BA content in HcD + CA-fed µMPs was higher than that in NcD- and HcD-fed µMPs (Table 2).

**Serum NOx and TBARS Levels**

The serum NOx levels in HcD + CA-fed µMPs were significantly increased in comparison to NcD- and HcD-fed µMPs after eight weeks, while those of NcD- and HcD-fed µMPs were not significantly different (Fig. 2C). Similarly, the serum MDA levels in HcD + CA-fed µMPs were markedly higher than those in NcD- and HcD-fed µMPs (Fig. 3B). The TBARS levels were significantly different between HcD- and HcD + CA-fed µMPs (Fig. 2D).

**Histopathological Analyses of the Liver**

After eight weeks, the livers of HcD + CA-fed µMPs were enlarged, pale and yellowish in color, whereas those of NcD- and HcD-fed µMPs showed no remarkable change (upper panels in Fig. 3). H&E sections showed that microvesicular steatosis and inflammation were significantly accelerated in HcD + CA-fed µMPs in comparison to NcD- and HcD-fed µMPs (middle panels in Fig. 3 and Table 3). Furthermore, although hepatocyte ballooning was occasionally seen in the liver of HcD + CA-fed µMPs, the frequency was significantly higher than that in other two groups. Consequently, significant differences were observed in NAFLD scores between NcD- and HcD-fed µMPs and between HcD + CA-fed µMPs (NcD; 0.25 ± 0.25, HcD; 1.20 ± 0.20 and HcD + CA; 4.40 ± 0.60) (Table 3). Oil-red O stain demonstrated that the livers of HcD + CA-fed µMPs contained a larger number of small lipid droplets, which were diffusely deposited in both the hepatocytes and sinusoidal foamy Kupffer cells (middle panels in Fig. 3). Perivascular and pericellular fibrosis were rarely seen in the livers of any groups in combined E-M stain (lower panels in Fig. 3).

**Biochemical Analyses of the Liver**

Hepatic TG, FFA and CE contents were markedly increased in HcD + CA-fed µMPs (Table 3). Although the levels of hepatic BA in HcD- and HcD + CA-fed groups were significantly higher than those in NcD-fed µMPs, there were no significant differences between two groups. The serum levels of AST, ALT, γ-GTP and ALP were increased in HcD + CA-fed µMPs as compared to those in NcD- and HcD-fed µMPs (Table 3). The expression levels of hydroxymethylglutaryl-CoA reductase (HMGCR) and LDL receptor (LDLR) were markedly lower in the livers of HcD + CA-fed µMPs in comparison to those of NcD- and HcD-fed µMPs (Supplemental Fig. 2).

### Table 3. Hepatic histological scores and lipid, BA contents, and blood chemistry

|                          | NcD (n=4) | HcD (n=5) | HcD + CA (n=5) |
|--------------------------|-----------|-----------|---------------|
| Steatosis score          | 0.00 ± 0.00 | 0.40 ± 0.24 | 1.60 ± 0.24 **# |
| Inflammation score       | 0.25 ± 0.25 | 0.80 ± 0.20 | 1.80 ± 0.20 ***## |
| Ballooning score         | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.00 ± 0.32 **# |
| NAFLD score              | 0.25 ± 0.25 | 1.20 ± 0.20 ** | 4.40 ± 0.60 ***## |
| Hepatic TG (mg/g)        | 4.2 ± 0.6  | 13.9 ± 1.4 *** | 23.3 ± 0.3 ***## |
| Hepatic FFA (µEq/g)      | 3.5 ± 0.3  | 7.6 ± 0.6 **  | 13.8 ± 0.6 ***## |
| Hepatic CE (mg/g)        | 3.3 ± 0.4  | 25.7 ± 1.6 *** | 30.6 ± 1.7 ***# |
| Hepatic BA (µM/g)        | 0.3 ± 0.02 | 0.5 ± 0.08 *  | 0.6 ± 0.20 * |
| AST (IU/L)               | 41.3 ± 4.4 | 33.0 ± 1.2 *  | 50.5 ± 3.0 ***## |
| ALT (IU/L)               | 44.7 ± 6.3 | 33.3 ± 1.4 *  | 52.8 ± 9.2 # |
| γ-GTP (IU/L)             | 50.3 ± 2.4 | 53.6 ± 2.1  | 74.0 ± 3.0 ***## |
| ALP (IU/L)               | 685.8 ± 100.7 | 983.2 ± 135.1 | 1006.6 ± 68.4 * |

Values are means ± SE. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. NcD-fed µMPs; and #P < 0.05, ##P < 0.01 and ###P < 0.001 vs. HcD-fed µMPs.
Fig. 4. Hepatic oxidative stress markers and apoptotic activity.

A) IHC revealed that very small numbers of liver cells accumulated 4-HNE and 8-OHdG in NcD- and HcD-fed µMPs. The 4-HNE and 8-OHdG-positive cell numbers were increased in HcD+CA feeding (arrowheads). Furthermore, the numbers of DHE-positive hepatocytes in the livers of HcD+CA-fed µMPs (red-stained; arrows) were increased than in NcD- and HcD-fed µMPs after eight weeks of treatment. C = central vein. B) Significantly greater numbers of TUNEL-positive hepatocytes (green-stained; arrows) were observed in HcD+CA-fed µMPs in comparison to NcD- and HcD-fed µMPs. The apoptotic hepatocytes occasionally showed condensed chromatin and fragmented nuclei with DAPI staining (inset). The values represent mean ± SE. *p < 0.05 and **p < 0.01 vs. NcD-fed µMPs; and #p < 0.05 and ##p < 0.01 vs. HcD-fed µMPs.

Oxidative Stresses and Apoptosis in the Livers

The numbers of liver cells that accumulated 4-HNE or 8-OHdG, which were low in NcD- and HcD-fed µMPs, were significantly increased in HcD+CA group (Fig. 4A). Correspondingly, significantly larger numbers of 4-HNE-positive and 8-OHdG-positive hepatocytes were observed in the livers of HcD+CA-fed µMPs (Fig. 4A). The numbers of DHE-positive hepatocytes of NcD- and HcD-fed µMPs were quite small, while there was a significant increase in those in the nuclei of livers of HcD+CA-fed µMPs (Fig. 4A). By TUNEL assay, HcD+CA-fed µMPs showed significantly increased numbers of apoptotic hepatocytes in comparison to NcD- and HcD-fed µMPs (Fig. 4B). These apoptotic hepatocytes occasionally showed condensed chromatin and fragmented nuclei (Fig. 4B).

Activated Kupffer Cells and Stellate Cells in the Livers of HcD+CA-fed µMPs

The livers of HcD+CA-fed µMPs contained increased numbers of lysozyme-positive sinusoidal macro-
The jejunal villi length in HcD + CA-fed µMPs was significantly increased in comparison to NcD- and HcD-fed µMPs (Supplemental Fig. 3A). No accumulation of small lipid droplets in the enterocytes was observed in any three groups. The expression levels of NPC1L1 were significantly lower in the jejunum of HcD- and HcD + CA-fed µMPs in comparison to NcD-fed group. However, no significant differences were detected between HcD- and HcD + CA-fed µMPs (Supplemental Fig. 3B).

**Discussion**

Some studies have been reported to develop swine models for NAFLD or metabolic syndrome[22-24). At present, however, a small number of studies have been reported to investigate the potential role of CA in the development of NAFLD using swine model[25). In the present study, in comparison with HcD feeding, HcD + CA feeding enhanced visceral adiposity, progression of NAFLD, and atherosclerosis in µMPs. To our knowledge, this is the first large animal model, in which visceral obesity, NAFLD and atherosclerosis are comcomitantly induced by eight-week dietary manipulation.

CA supplementation to HcD accompanied higher phagocytes with foamy cytoplasm (Kupffer cells) than those of NcD- and HcD-fed groups (Fig. 5A). In addition, fibrogenesis and stellate cell activation, as determined by IHC for α-SMA, were not apparent in NcD- and HcD-fed µMPs (Fig. 6B). In contrast, IHC demonstrated a linear expression of α-SMA along the hepatic sinusoids of HcD + CA-fed µMPs. This was accompanied by increased numbers of activated hepatic stellate cells in HcD + CA-fed µMPs (Fig. 5B).

**Systemic Mobilization of Foamy Macrophages in HcD + CA-fed µMPs**

The reticuloendothelial system (RES), including bone marrow, spleen and lymph nodes, contained an inflammatory lesion consisting of a collection of foamy macrophages in HcD + CA-fed µMPs compared with NcD-fed groups (Fig. 6). The lesions in bone marrow and lymph nodes were very often found in HcD + CA-fed µMPs (Table 4). In addition, an accumulation of foamy macrophages was observed in the capillary vessels of alveolar septa (pulmonary intravascular macrophages; PIM) and the upper dermis of the skin (cutaneous xanthoma cells) only in HcD + CA-fed µMPs, but not in other two groups (Fig. 7 and Table 4).

**The Histological and Molecular Analyses of Small Intestine**

The jejunal villi length in HcD + CA-fed µMPs was significantly increased in comparison to NcD- and HcD-fed µMPs (Supplemental Fig. 3A). No accumulation of small lipid droplets in the enterocytes was observed in any three groups. The expression levels of NPC1L1 were significantly lower in the jejunum of HcD- and HcD + CA-fed µMPs in comparison to NcD-fed group. However, no significant differences were detected between HcD- and HcD + CA-fed µMPs (Supplemental Fig. 3B).
levels of serum oxidative stress markers, TNF-α and BA levels, and systemic foamy macrophage mobilization but no significant difference in serum lipid levels. In addition, increased hepatic TG, FFA, BA and oxidative stresses, and accelerated activation of sinusoidal foamy Kupffer cells and stellate cells, along with enhanced hepatocyte apoptosis, were observed in the livers of HcD+CA-fed µMPs, indicating that CA induces progression of NAFLD. Furthermore, increased numbers of foamy macrophages were identified in various organs, such as the RES, including bone marrow, liver, spleen and lymph nodes, as well as the pulmonary capillary vessels and skin in HcD+CA-fed µMPs. These results provide novel insights into the effects of CA on systemic macrophage activation possibly through oxidative stress, TNF-α signaling and progression of visceral adiposity, NAFLD and atherosclerosis. The administration of CA might augment the local (intrahepatic) and systemic (circulating) expression of oxidative stressors and TNF-α, and stimulate macrophages mobilization. These would accelerate visceral obesity, atherosclerosis, hepatic steatosis, inflam-

Fig. 6. Histological analyses of mobilization of foamy macrophages in the reticuloendothelial system.

H&E sections demonstrated that the reticuloendothelial system, including the liver (as shown in Fig. 3), bone marrow (A, B), spleen (C, D), and lymph nodes (E, F) from HcD+CA-fed µMPs contained increased numbers of infiltrating foamy macrophages in comparison to NcD- and HcD-fed groups. Foamy macrophages were infiltrating in the sinuses in the spleen and lymph node. A) C) E), low power magnification; B) D) F), high power magnification.
showed inflammation, macrophage, and stellate cell activation, steatosis and apoptotic cell death of hepatocytes but did not meet the full requirements of diagnosis for NASH because of the absence of pericellular sinusoidal fibrosis. The liver tissues, however, contained increased amounts of cholesterol, TG, and FFA, which is considered to be a first step for the development of NAFLD. On the basis of alteration of hepatic lipid metabolism, a second hit is required to promote oxidative stress that overwhelms the endogenous cell survival mechanisms, leading to hepatocyte apoptosis, fibrosis, and cirrhosis. CA and CA-related BAs (e.g., secondary BAs such as deoxy-CA and ursodeoxy-CA) function as signaling molecules for the induction of oxidative stress, and BA levels including deoxy-CA and CA are actually increased in the NASH liver tissues of human patients. Thus, CA would play a certain role in the pathogenesis of NAFLD in the present swine model and recently reported rat model, in which a high-fat/high-cholesterol diet with 2% CA induces NASH-related cirrhosis. Another swine model using Ossabaw miniature swine showed that 0.7% CA (equal to HcD-CA diet composition in the present study) contained atherogenic diet-induced microvesicular steatosis, Kupffer cell activation and hepatocyte ballooning but not inflammation or fibrosis. In this Ossabaw miniature swine, modification of the atherogenic diet (different source of fat and higher protein but lower choline content) is necessary to develop hepatic inflammation and fibrosis. It is still unclear why the different sensitivity to CA is observed in the different animal models, but some studies suggested that the source of protein in the diet affects pancreatic hormone secretion and lipid metabolism-related hepatic gene expression, which may modify the hepatic lesion development.

In the studies of atherosclerosis animal models, especially using non-gene-modified mice and rabbits, CA is often supplemented with high-fat/cholesterol diet to enhance hypercholesterolemia by interfering with hepatobiliary excretion or by increased intestinal absorption of cholesterol, resulting in atherosclerosis. On the other hand, CA is not required for the development of atherosclerosis in LDLR-deficient mice, like in the swine model using μMP as previously reported. In the LDLR-deficient mice, the atherosclerotic lesion area of the aorta was not enhanced by a CA diet (40% fat, 1.25% cholesterol, and 0.5% cholic acid) compared with a CA-free diet (40% fat and 1.25% cholesterol), even though hypercholesterolemia was induced by CA diet. This is a result to the contrary in the present study that HcD + CA-fed μMPs showed accelerated atherosclerosis, but hypercholesterolemia was not enhanced compared with CA-free HcD-fed μMPs. Since the jejunum NPC1L1, a cholesterol-uptake transporter, and hepatic HMGCR and LDLR expression were markedly suppressed to the same degree in HcD- and HcD + CA-fed μMPs, CA would have no more effects to enhance hypercholesterolemia mediated through the regulation of these cholesterol-related genes. This indicate a presence of another potential mechanism(s) of dietary CA for the progression of atherosclerosis, not by a further induction of hypercholesterolemia. One possible explanation for the mechanism(s) is that CA stimulates vascular response to induce the progression of atherosclerosis via induction of oxidative stress and inflammation. However, the serum lipid levels of HcD-CA-fed μMPs showed a borderline significant trend in comparison with those of HcD-fed animals, it seems difficult to completely exclude the possibility that the effect of CA supplementation is totally independent of serum lipid profiles.

The hepatic histology of HcD + CA-fed μMPs showed inflammation, macrophage, and stellate cell activation, steatosis and apoptotic cell death of hepatocytes but did not meet the full requirements of diagnosis for NASH because of the absence of pericellular sinusoidal fibrosis. The liver tissues, however, contained increased amounts of cholesterol, TG, and FFA, which is considered to be a first step for the development of NAFLD. On the basis of alteration of hepatic lipid metabolism, a second hit is required to promote oxidative stress that overwhelms the endogenous cell survival mechanisms, leading to hepatocyte apoptosis, fibrosis, and cirrhosis. CA and CA-related BAs (e.g., secondary BAs such as deoxy-CA and ursodeoxy-CA) function as signaling molecules for the induction of oxidative stress, and BA levels including deoxy-CA and CA are actually increased in the NASH liver tissues of human patients.

Thus, CA would play a certain role in the pathogenesis of NAFLD in the present swine model and recently reported rat model, in which a high-fat/high-cholesterol diet with 2% CA induces NASH-related cirrhosis. Another swine model using Ossabaw miniature swine showed that 0.7% CA (equal to HcD-CA diet composition in the present study) contained atherogenic diet-induced microvesicular steatosis, Kupffer cell activation and hepatocyte ballooning but not inflammation or fibrosis. In this Ossabaw miniature swine, modification of the atherogenic diet (different source of fat and higher protein but lower choline content) is necessary to develop hepatic inflammation and fibrosis. It is still unclear why the different sensitivity to CA is observed in the different animal models, but some studies suggested that the source of protein in the diet affects pancreatic hormone secretion and lipid metabolism-related hepatic gene expression, which may modify the hepatic lesion development.

The HcD + CA-fed μMPs showed a unique phenotype of systemic macrophage mobilization, which was characterized by a significant increase in the num-

Table 4. Summary of histological findings

|                      | NcD   | HcD   | HcD + CA   |
|----------------------|-------|-------|------------|
|                      | n=4   | n=5   | n=5        |
| Atherosclerosis      |       |       |            |
| Foamy Mφ infiltration|       |       |            |
| Bone marrow          | 0/4   | 5/5   | 5/5        |
| Spleen               | 0/4   | 2/5   | 5/5        |
| Lymph node           | 0/4   | 4/5   | 5/5        |
| Lung                 | 0/4   | 1/5   | 5/5        |
| Skin                 | 0/4   | 0/5   | 4/5        |
| Frequency            | +     | +     | +          |
|                      | +     | +     | +          |
|                      | +     | +     | +          |
|                      | +     | +     | +          |
|                      | +     | +     | +          |
|                      | +     | +     | +          |
|                      | -     | +     | +          |

In the studies of atherosclerosis animal models, especially using non-gene-modified mice and rabbits, CA is often supplemented with high-fat/cholesterol diet to enhance hypercholesterolemia by interfering with hepatobiliary excretion or by increased intestinal absorption of cholesterol, resulting in atherosclerosis. On the other hand, CA is not required for the development of atherosclerosis in LDLR-deficient mice, like in the swine model using μMP as previously reported. In the LDLR-deficient mice, the atherosclerotic lesion area of the aorta was not enhanced by a CA diet (40% fat, 1.25% cholesterol, and 0.5% cholic acid) compared with a CA-free diet (40% fat and 1.25% cholesterol), even though hypercholesterolemia was induced by CA diet. This is a result to the contrary in the present study that HcD + CA-fed μMPs showed accelerated atherosclerosis, but hypercholesterolemia was not enhanced compared with CA-free HcD-fed μMPs. Since the jejunum NPC1L1, a cholesterol-uptake transporter, and hepatic HMGCR and LDLR expression were markedly suppressed to the same degree in HcD- and HcD + CA-fed μMPs, CA would have no more effects to enhance hypercholesterolemia mediated through the regulation of these cholesterol-related genes. This indicate a presence of another potential mechanism(s) of dietary CA for the progression of atherosclerosis, not by a further induction of hypercholesterolemia. One possible explanation for the mechanism(s) is that CA stimulates vascular response to induce the progression of atherosclerosis via induction of oxidative stress and inflammation. However, the serum lipid levels of HcD-CA-fed μMPs showed a borderline significant trend in comparison with those of HcD-fed animals, it seems difficult to completely exclude the possibility that the effect of CA supplementation is totally independent of serum lipid profiles.
numbers of foamy macrophages. These macrophages can be more frequently identified in various organs from HcD + CA-fed µMPs, such as RES, including bone marrow, liver, spleen, and lymph nodes, as well as the lung and upper dermis. In the lung, macrophage accumulation in the capillary vessels of alveolar septum is known as PIMs, which are normally found in selected species including swine, but not often found in humans. The PIM is classified into two different conditions: constitutive and induced PIM. The constitutive PIM has been observed in swine, sheep, goats, and horse, and induced PIM has been reported in rats, mice, guinea pigs, and hamsters. After operation of the common bile duct ligation, chronic bile duct obstruction induces PIM in rats. Other studies have suggested that the depletion of induced PIM in bile-ligated rats reduced endotoxin-induced pulmonary inflammation. The bile duct ligation also increased endotoxin sensitivity, however, lipopolysaccharide alone cannot explain the induction of PIM. Thus, increased serum and hepatic BAs, due to chronic bile duct obstruction and impaired BA excretion might stimulate systemic and hepatic oxidative response and pro-inflammatory cytokine production to recruit macrophages in the RES. This situation would be very similar to the present studies that dietary CA induces oxidative and inflammatory responses to systemically activate the RES.

**Conclusion**

HcD + CA-fed µMPs exhibited unique features of visceral adiposity, progressed NAFLD and atherosclerosis and systemic macrophage mobilization. The present study provides new evidence of a possible mechanism(s) by which CA increases both local and systemic expression of oxidative stress and inflammatory cytokine, and activates macrophages in various organs, promoting visceral obesity, atherosclerosis and NAFLD.

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The authors declare no conflicts of interest in association with the present study.

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Supplemental Table 1. Primers used for real-time RT-PCR

| Gene | Assay ID       | Custom made primers                      | RefSeq      | GenBank     |
|------|----------------|------------------------------------------|-------------|-------------|
| LDLR | Ss03374441_ul  |                                          | NM_001206354.1 | AF065990.1 |
| HMGCR| Ss03390147_mL |                                          | NM_001122988.1 | DQ432054.1 |
| NPC1L1|              | Forward: CCTGTTCGAGCGAGGTCTCTTA          | XM_003134893.1 |             |
|      |               | Reverse: GAAAGAGGAATAGTCGAGCAGGTA        |             |             |
|      |               | Probe: CTGCCCAAGGACTC                    |             |             |

The assay IDs are listed for the predesigned gene expression assay (ABI). The primers and probes for the NPC1L1 analyses were created based on swine sequences.

Supplemental Fig. 1. Serum inflammatory cytokines levels after 8 week feeding.

The serum levels of TNF-α were moderately increased in HcD-CA-fed µMPs after 8 week feeding, but not those of IL-1β and IL-6 was not detected in our experimental conditions. (*) The increase of TNF-α showed a possible trend toward significance (p=0.0569) vs. NcD-fed µMPs.
Supplemental Fig. 2. Cholesterol metabolism-related gene expression in the liver.

The real-time RT-PCRs demonstrated that expression levels of NPC1L1 in the livers of HcD + CA-fed µMPs were lower in comparison to NcD- and HcD-fed µMPs. Moreover, expression levels of HMGCR and LDLR in the livers of HcD + CA-fed µMPs were lower than those in the livers of NcD- and HcD-fed µMPs. The values represent mean ± SE and were normalized to the 18S rRNA expression. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. NcD-fed µMPs; and °p < 0.05, °°p < 0.01 and °°°p < 0.001 vs. HcD-fed µMPs.

Supplemental Fig. 3. Histological and biochemical analyses of the jejunum.

A) The jejunal villi length in HcD + CA µMPs was increased in comparison to that in NcD- and HcD-fed µMPs. Accumulation of small lipid droplets in the surface enterocytes was not observed in any µMPs. B) A real-time RT-PCR showed that expression levels of NPC1L1, one of key cholesterol-uptake transporters, in the jejunum of HcD- and HcD + CA-fed µMPs were lower in comparison to NcD-fed µMPs. This suggested a negative feedback process against pronounced cholesterol/BA absorption and transport. However, there were no significant differences between HcD- and HcD + CA-fed µMPs. The values represent mean ± SE and were normalized to 18S rRNA expression. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. NcD-fed µMPs; and °p < 0.05, °°p < 0.01 and °°°p < 0.001 vs. HcD-fed µMPs.