Original Article

Analysis of vanin-1 upregulation and lipid accumulation in hepatocytes in response to a high-fat diet and free fatty acids

Wataru Motomura,[1] Takayuki Yoshizaki,[2,*] Nobuhiko Takahashi,[3] Shima Kumei,[4] Yusuke Mizukami,[3] Seong-Jae Jang[1] and Yutaka Kohgo[3]

[1]Department of Microbiology and Immunochemistry, [3]Department of Medicine, Division of Gastroenterology and Hematology/Oncology and [4]Department of Medicine, Division of Gastroenterology and Pharmacology, Asahikawa Medical College, 2-1-1-1 Midorigaoka-Higashi, Asahikawa, Hokkaido 078-8510, Japan
[2]Innovation Center, Kagoshima University, 1-21-40 Korimoto, Kagoshima 890-0065, Japan

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High-fat diet is one of the causes of nonalcoholic fatty liver disease. We have previously demonstrated that high-fat diet induces upregulation of adipose differentiation-related protein mRNA expression accompanied by lipid droplet formation in mouse liver. Vanin-1 is a ubiquitous epithelial ectoenzyme that has pantetheinase activity and produces cysteamine, a potent endogenous antioxidant. In the present study, we analyzed the expression of hepatic vanin-1 mRNA following the administration of a high-fat diet in mice as well as free fatty acids in hepatocyte cultures and speculated its possible mechanism. Vanin-1 mRNA levels in the livers of mice were upregulated within a day of the high-fat diet, even before the expression of adipose differentiation-related protein mRNA and lipid accumulation. An *in vitro* analysis using HuH-7 cells revealed a significant upregulation of vanin-1 mRNA by as low as 0.01 mM oleic acid; however, lipid accumulation in hepatocytes was not affected at this concentration. Furthermore, vanin-1 mRNA was differentially upregulated by various free fatty acids irrespective of the grade of lipid accumulation. These findings indicate that the upregulation of vanin-1 precedes lipid accumulation and is differentially mediated by various types of free fatty acids in the model, presenting vanin-1 as a novel player in the pathogenesis of nonalcoholic fatty liver disease.

***Key Words*:** **fatty acids, lipid droplet, nonalcoholic fatty liver disease, vanin-1**

Hepatic cells are important stores of neutral lipids that act as a physiological buffer. Although lipid storage is necessary for energy preservation and synthesis of lipoproteins and steroid hormones, excess accumulation often leads to disorders such as fatty liver, obesity, and atherosclerosis.[1,2] Nonalcoholic fatty liver disease (NAFLD) covers a wide array of pathological conditions, ranging from benign fatty liver to different forms of nonalcoholic steatohepatitis (NASH) including steatosis with inflammation, steatosis with inflammation and mild-to-advanced fibrosis, steatosis with fibrosis alone, cirrhosis, and end-stage liver disease.[3,4] NASH is a more severe histological form of NAFLD, and has gained clinical importance in developing countries due to its progression to cirrhosis.[3,4] A "two-hit" hypothesis has been suggested for the pathogenesis of NAFLD.[5] The "first hit" is caused by fat deposition in the liver that is closely associated with insulin resistance, leading to NAFLD. The "second hit" is a result of oxidative stress, leading to lipid peroxidation and increased cytokine production and inflammation in the hepatocytes, ultimately resulting in NASH.[6]

Currently, the mechanism for the development of hepatic steatosis is being elucidated through the identification of genes involved in lipid metabolism using mouse models of fatty liver disease. In these studies, vanin-1 has been recognized as a candidate for lipid metabolism-related genes. Vanin-1 gene expression was upregulated 17.1-fold in peroxisome proliferator-activated receptor alpha-deficient (PPAR$\alpha^{-/-}$) mice who overexpressed PPARγ when compared with fatty livers induced by fasting or choline deficiency.[7] Similarly, DNA microarray analysis of phosphatase and tensin homolog-deficient (Pten$^{-/-}$) mice exhibiting hepatic steatohepatitis revealed a 3-fold upregulation of the vanin-1 gene when compared with their non-fatty liver counterparts.[8] Furthermore, vanin-1 mRNA expression in apolipoprotein E-deficient (apoE$^{-/-}$) mice, in whom fatty liver was accelerated by administering *trans*-10, *cis*-12-conjugated linoleic acid, was demonstrated to be higher than that in *trans*-11, *cis*-9 linoleic acid-administered mice exhibiting amelioration of fatty liver. In this report, the vanin-1 gene was considered to be associated with fatty liver based on its high steatotic index.[9] These studies clearly raise the possibility of an association between vanin-1 and the pathologic course of fatty liver disease.

Vanin-1 is an ectoenzyme anchored to the surface of epithelial cells by a glycosylphosphatidyl inositol moiety.[10] Its pantetheinase activity is involved in the metabolic pathway of pantothenate (vitamin B5) and is a main provider of cysteamine to tissues.[11] Interestingly, both the presence and absence of vanin-1 has been demonstrated to have a cytoprotective role in different cell types. Vanin-1 deficient mice show elevated glutathione (GSH) levels that are associated with better resistance to oxidative injury and with reduced apoptosis, suggesting that vanin-1 may be a negative regulator of cellular GSH storage.[12] Further, vanin-1-deficient mice show a remarkably increased resistance to stress and decreased intestinal inflammation.[13,14] Epithelial vanin-1 was also found to regulate inflammation-driven cancer development in a colitis-associated colon cancer model.[15] The presence of mouse vanin-1 has been shown to play a cytoprotective role for islet beta cells possibly through the antioxidant property of cysteamine, and shown to regulate the development of type 1 diabetes. It has been suggested that the impact of cysteamine on a disease state may directly depend upon local tissue concentrations, although the mechanisms of control of inflammation vs survival by cysteamine are poorly characterized.[16] In the light of these studies, we hypo-

*To whom correspondence should be addressed.
E-mail: k8255261@kadai.jp

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thesized that the regulation of vanin-1 may determine the progression of fatty liver disease.

Although microarray studies in PPARα$^{-/-}$, Pten$^{-/-}$, and apoE$^{-/-}$ mice have demonstrated the upregulation of vanin-1 in the lipid metabolic pathway in the liver,[7–9] the contribution of vanin-1 to the development of hepatic steatosis is not known. Therefore, the aim of this study was to evaluate the induction of the vanin-1 gene in obese mice as well as in human hepatocyte cells treated with various fatty acids.

Materials and Methods

Animals, biochemical parameters, and tissue analyses. Nine-week-old male C57Bl/6N and C57Bl/6J-*ob/ob* (*ob/ob*) mice, purchased from Sankyo Labo Service (Tokyo, Japan), were housed at 22°C under a 12-h light-dark cycle (lights on at 0700 h) and were allowed ad libitum access to food and water. Blood glucose was measured using Glucocard (Arkray, Kyoto, Japan). Serum parameters were measured using a Clinical Analyzer 7180 (Hitachi High-Technologies, Tokyo, Japan). For histology and immunohistochemistry, the mice were anesthetized with 2.5% avertin and perfused through the left ventricle with 20 mL of ice-cold PBS and then with 4% paraformaldehyde in PBS. Liver tissue was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and the sections stained with hematoxylin and eosin (HE) for histopathologic evaluation. All the experiments were conducted in accordance with the rules and guidelines of the Animal Experiment Committee of Asahikawa Medical College.

***In situ* hybridization.** *In situ* hybridization was performed using a digoxigenin (Roche Molecular Biochemicals, Mannheim, Germany)-labeled copy RNA (cRNA) probe for vanin-1 mRNA as described previously.[17]

Cell culture and histopathological lipid-droplet evaluation. Human hepatocellular carcinoma cell line HuH-7 was obtained from RIKEN BioResource Center (Ibaraki, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), and incubated at 37°C in a humidified atmosphere of 5% CO_2. Free fatty acids (FFAs) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.1 M and stored at −20°C before use. In some cultures, fatty acids (as a sodium salt, conjugated with 2% bovine serum albumin (BSA) and/or other reagents were added to the medium at the indicated concentrations. HuH-7 cells were plated in glass-bottom culture dishes (Matsunami Glass, Osaka, Japan) and cultured in DMEM containing 10% FBS. The medium was changed to DMEM containing fatty acids conjugated with BSA and 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503; Invitrogen) as a lipid probe. The cells were fixed with 4% paraformaldehyde, permeabilized, and then incubated with TO-PRO-3 (Invitrogen) for nuclear staining. Fluorescent images were observed with a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan).

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. Real-time RT-PCR analyses were performed as described previously.[18,19] Briefly, total RNA was extracted using TRIzol reagent (Invitrogen). Complementary DNA was synthesized using a RETROscript kit (Applied Biosystems/Ambion, Austin, TX). Real-time PCR analysis was performed using an Applied Biosystems 7300 Real-Time PCR System (Foster City, CA) according to the manufacturer's specifications. TaqMan probes for mouse vanin-1 (Mm00495965_m1), human vanin-1 (Hs00190982_m1), human PPARα (Hs00947539_m1), and human adipose differentiation-related protein (ADRP; Hs00605340_m1) were purchased from Applied Biosystems. To normalize the relative expression of the genes of interest, eukaryotic 18S rRNA (Hs99999901_s1, X03205.1) was used as an endogenous control.

Statistical analysis. Statistical analysis was performed either by 1-way analysis of variance along with the Tukey multiple comparison test or by 2-way analysis of variance with subsequent Bonferroni post-test. All tests were performed using GraphPad Prism ver. 5 (GraphPad Software, La Jolla, CA). $p<0.05$ was considered significant.

Results

Vanin-1 mRNA expression following the high-fat diet. Fig. 1A shows the relative vanin-1 mRNA expression in the livers of mice fed with the high-fat diet for 2, 4, 6, and 12 weeks. Vanin-1 mRNA was upregulated in the high-fat diet groups compared with the normal diet groups starting from the early disease stage, although there was no significant difference between the 2-week and the 12-week groups, indicating that vanin-1 mRNA expression was not regulated by the duration for which the high-fat diet was administered. In a previous study, we observed that normal mice fed with a high-fat diet for 2 to 6 weeks did not develop diabetes; however, liver steatosis was confirmed as early as 2 weeks through the detection of triacylglycerol (TG) accumulation by Oil Red O staining.[20] To determine a possible association between the onset of high-fat diet-induced steatosis and vanin-1 mRNA expression, we focused on the vanin-1 mRNA expression in the first week of high-fat dietary administration. Although liver steatosis did not occur within the first week (H&E staining; data not shown) in mice on a high-fat diet, vanin-1 mRNA expression was increased from Day 1 when compared to that in Day 0 control mice (Fig. 1B). Vanin-1 mRNA expression level had returned to control level when the mouse fed the normal diet for 4 days following the high-fat diet for 3 days (Day 3 + 4).

Effects of the high-fat diet condition on physiological and biochemical parameters. Total cholesterol (T-Cho), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and FFA levels were significantly elevated in the high-fat diet groups accompanied by an increase in body weight, liver weight, and white adipose tissue (WAT) (Table 1). There were no significant differences between Day 3 + 4 and Day 0 control mice, except glutamic-pyruvic transaminase (GPT) level. These results indicated that FFA uptake to the liver was higher in the high-fat diet group, which possibly affected lipid metabolism and vanin-1 mRNA expression in the hepatocytes of these mice.

Steatosis in *ob/ob* mice and vanin-1 mRNA expression in *ob/ob* mouse livers. Spontaneously obese (*ob/ob*) mice have been used as an effective model to understand the mechanisms leading to the development of hepatic steatosis.[21] The grade of liver steatosis gradually advanced in the 14- and 32-week-old mice (Fig. 2A). The relative expression of vanin-1 mRNA in the livers of 14- and 32-week *ob/ob* mice was approximately 6–7 times higher than in the livers of wild type mice (Fig. 2B). The expression of vanin-1 mRNA between the 2 age groups was not significantly different, although the grade of liver steatosis gradually advanced (Fig. 2A).

Hepatic distribution of vanin-1 mRNA by *in situ* hybridization. In 34-week-old *ob/ob* mice, vanin-1 mRNA was expressed strongly and particularly in hepatocytes near large lipid droplets in the central vein area (Fig. 3 A and B). These results suggest that the expression of vanin-1 mRNA is more prominent in the areas of lipid droplet formation in the liver.

Effect of FFAs on lipid accumulation and *in vitro* expression of hepatic vanin-1 mRNA. The high-fat diet fed to the experimental mice comprised of 58% lard obtained from pigs, and oleic acid constitutes about 44–47% of the FFAs in the lard.[22] So we used the hepatoma cell line HuH-7 as an *in vitro* lipid-accumulation model to investigate the direct effect of oleic acid in hepatocytes. Oleic acid concentrations higher than 1 mM induced lipid droplet formation, whereas 0.1 and 0.01 mM were ineffective

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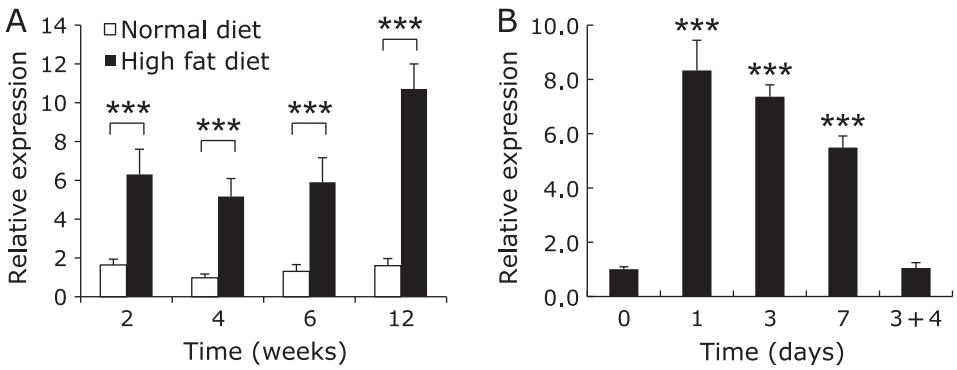

Fig. 1. Vanin-1 mRNA expression in mouse liver with steatosis induced by the high-fat diet. (A) Relative expression of vanin-1 in normal chow-fed and high-fat diet-fed mice. The values shown are the mean (SEM) (n = 5); ***p<0.001 by analysis of variance compared with the normal diet. (B) Relative expression of mouse vanin-1 induced by the high-fat diet during the first week (Day 1–Day 7). "Day 3 + 4" indicates a normal diet was fed for 4 days after feeding a high-fat diet for 3 days. The data were normalized to the value of 18S rRNA. The values shown are the mean (SEM) (n = 5); ***p<0.001 by analysis of variance compared with Day 0 (prior to induction by high-fat diet).

Table 1. Effects of the high-fat diet condition on physiological and biochemical parameters

Parameter	Day 0	Day 1	Day 3	Day 7	Day 3 + 4
Body weight (g)	22.6 ± 0.5	23.6 ± 0.8	25.5 ± 0.5*	25.3 ± 0.5*	23.6 ± 0.7
Liver weight (g)	1.26 ± 0.05	1.34 ± 0.06	1.52 ± 0.05*	1.44 ± 0.05	1.24 ± 0.07
WAT weight (g)	0.24 ± 0.04	0.34 ± 0.05	0.46 ± 0.04*	0.44 ± 0.06*	0.28 ± 0.02
Blood sugar (mg/dL)	175 ± 5	155 ± 6	195 ± 6	180 ± 13	179 ± 7
Total Protein (g/dL)	5.1 ± 0.1	5.5 ± 0.2	5.1 ± 0.1	5.0 ± 0.1	4.6 ± 0.0
Albumin (g/dL)	2.3 ± 0.1	2.4 ± 0.1	2.2 ± 0.0	2.2 ± 0.1	2.2 ± 0.0
T-Cho (mg/dL)	79 ± 3	120 ± 3***	197 ± 4***	178 ± 8***	77 ± 2
HDL-C (mg/dL)	44 ± 2	71 ± 3***	103 ± 2***	91 ± 6***	42 ± 1
LDL-C (mg/dL)	6 ± 0	11 ± 0**	16 ± 1***	17 ± 1***	6 ± 0
Triglyceride (mg/dL)	46 ± 7	107 ± 12	51 ± 16	94 ± 25	71 ± 9
FFA (mEq/l)	0.88 ± 0.11	2.06 ± 0.14***	1.51 ± 0.15	1.50 ± 0.22	0.92 ± 0.05
GPT (U/l)	12 ± 2	9 ± 1	9 ± 0	12 ± 2	4 ± 1**

The data are expressed as mean ± SEM (n = 5). *p<0.05, **p<0.01, and ***p<0.001 vs the controls. "Day 3 + 4" indicates a normal diet was fed for 4 days after feeding a high-fat diet for 3 days. WAT indicates white adipose tissue; T-Cho, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; FFA, free fatty acid; GPT, glutamic-pyruvic transaminase.

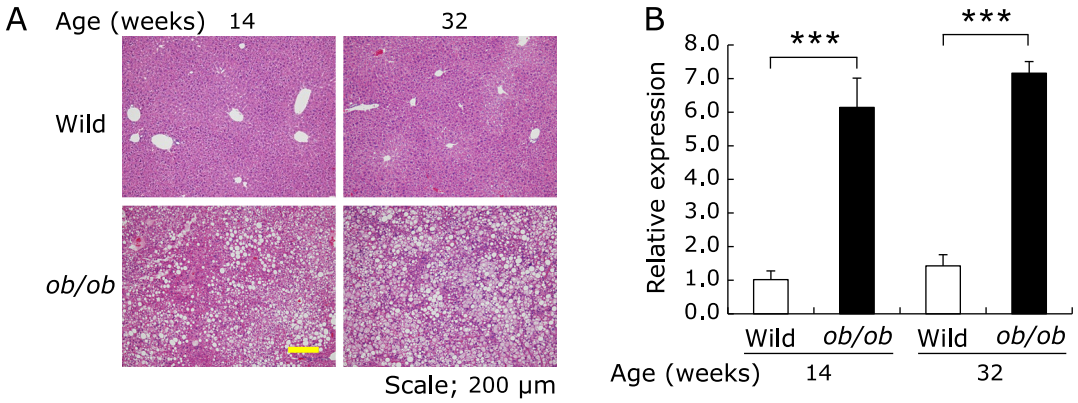

Fig. 2. Vanin-1 mRNA expression in *ob/ob* mouse liver. (A) Results of the H&E staining of tissues from 14- and 32-week-old wild-type and *ob/ob* mice. (B) Relative expression of vanin-1 in the wild-type and *ob/ob* mice. The data were normalized to the value of 18S rRNA used as an endogenous control. The values shown are the mean (SEM) (n = 5); ***p<0.001 by analysis of variance.

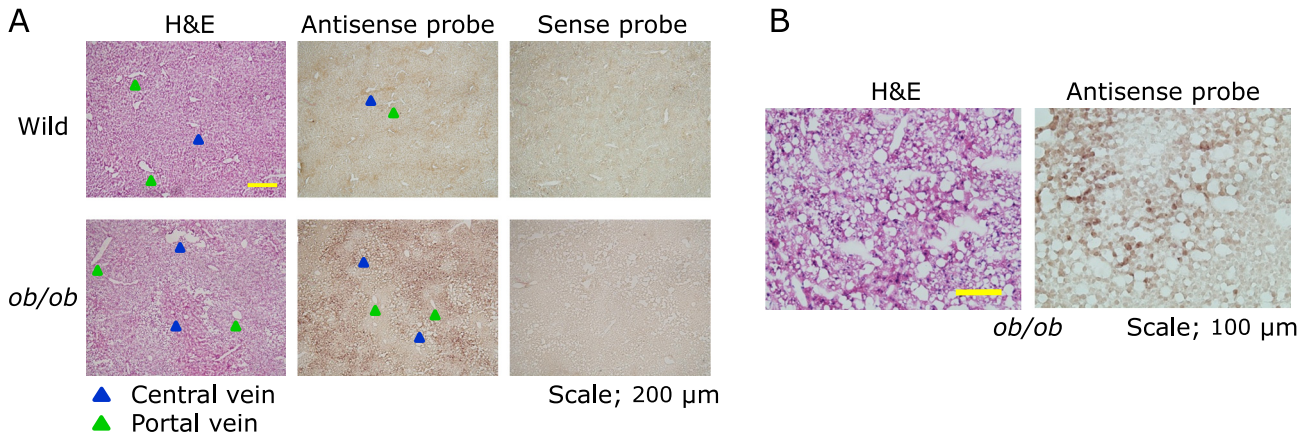

Fig. 3. Distribution of vanin-1 mRNA in steatotic liver tissues of *ob/ob* mouse. (A) *In situ* hybridization of tissues from wild-type and 32-week-old *ob/ob* mice. An antisense probe was used to detect vanin-1 mRNA and a sense probe was used as the negative control. (B) Right panels are high-magnification images of liver sections of *ob/ob* mice.

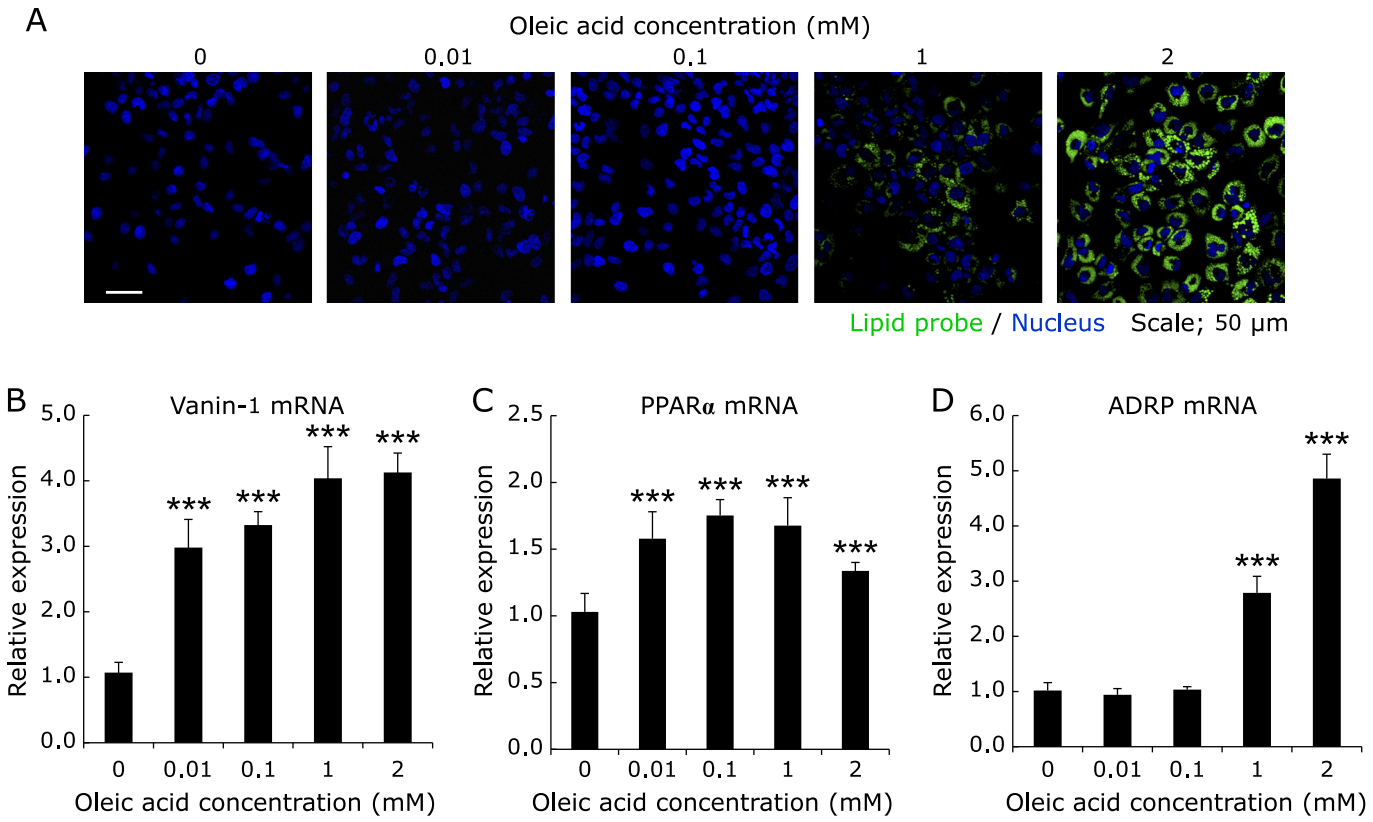

Fig. 4. Oleic acid (C18:1)-induced vanin-1 mRNA expression during the course of lipid accumulation in HuH-7 cells. (A) Immunofluorescence to show lipid accumulation (green) and nuclei (blue) in human hepatoma cell line HuH-7. After 24-h induction with 0 (control), 0.01, 0.1, 1, and 2 mM oleic acid, HuH-7 cells were fixed and observed by confocal laser-scanning microscopy. Note the absence of green fluorescence at oleic acid concentrations of 0.01 and 0.1 mM. (B)–(D) Relative expressions of vanin-1 (B), PPARα (C), and ADRP (D) in HuH-7 cells with the same doses of oleic acid. The values shown are the mean (SEM) (n = 6); ***p<0.001 by analysis of variance compared with the controls.

in inducing the same (Fig. 4A). Vanin-1 mRNA expression was significantly upregulated with concentrations of oleic acid ≥0.01 mM when compared with the controls (Fig. 4B); however, lipid droplets were not observed at oleic acid concentrations of 0.01 and 0.1 mM. Oleic acid upregulated PPARα mRNA expression when compared with the expression in control cultures (Fig. 4C), indicating that oleic acid act as a mediator to increase PPARα mRNA expression in HuH-7 cells. The mRNA expression of ADRP increased at oleic acid concentrations of 1 mM and above (Fig. 4D), which is consistent with the results shown in

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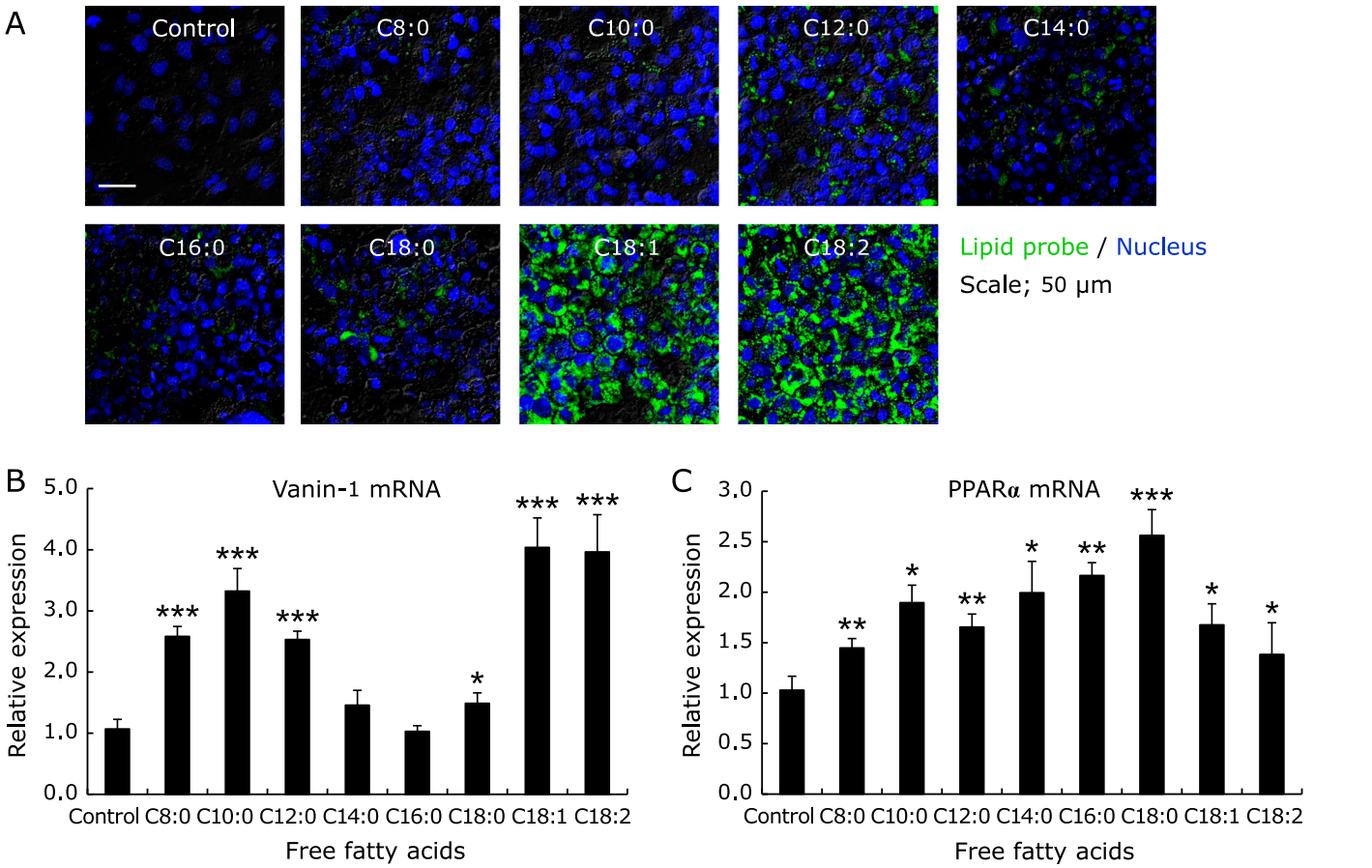

Fig. 5. Induction of vanin-1 mRNA expression by different FFAs in HuH-7 cells. "Control" indicates control cells without FFAs. (A) Immunofluorescence to show lipid accumulation (green) and nuclei (blue) in HuH-7 cells. After 24-h induction by FFAs, HuH-7 cells were fixed and observed by confocal laser-scanning microscopy. Note the abundance of green fluorescence for oleic acid (C18:1) and linoleic acid (C18:2). (B) and (C) Relative expressions of vanin-1 (B) and PPARα (C) in HuH-7 cells with different FFAs. The values shown are the mean (SEM) (n = 6); *p<0.05, **p<0.01, and ***p<0.001 by analysis of variance compared with the controls.

Fig. 4A. Additionally, lipid droplet formation was induced by oleic acid (C18:1) and linoleic acid (C18:2), but not by octanoic acid (C8:0), decanoic acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), and stearic acid (C18:0) (Fig. 5A). Further, when compared with the control cultures, the upregulation of vanin-1 mRNA was extremely significant with octanoic acid (C8:0), decanoic acid (C10:0), lauric acid (C12:0), and linoleic acid (C18:2), and moderately significant with stearic acid (C18:0), but insignificant with myristic acid (C14:0) and palmitic acid (C16:0) (Fig. 5B). Meanwhile, all FFAs used in this study upregulated PPARα mRNA expression when compared with the expression in control cultures (Fig. 5C). These results suggested that the regulation of vanin-1 mRNA expression depended on chain length and nature of bonds in the FFAs.

Discussion

In the present study, we demonstrated that levels of vanin-1 mRNA in mice on a high-fat diet increase within a day and that vanin-1 mRNA expression is more prominent around lipid droplet formations in the liver. Although vanin-1 expression did not change with the age of the mice with liver steatosis or grade of liver steatosis, the appearance of vanin-1 upregulation before the onset of liver steatosis suggests a definite role of vanin-1 in the progression of fatty liver disease.

Our findings indicate that serum FFAs derived from a high-fat diet may act as regulators of vanin-1 mRNA expression. Normally, high plasma FFA levels may either increase hepatic FFA uptake or at least maintain a normal rate of hepatic FFA uptake, despite increased hepatic FFA levels.[23] Therefore, in our study model, the vanin-1 mRNA expression in hepatocytes may have been upregulated by the increased rate of hepatic FFA uptake caused by elevated plasma FFA levels.

In general, FFAs are continuously metabolized and oxidized in hepatocytes. Oxidative products generated during these processes give rise to oxidative stress.[24,25] Vanin-1 gene expression is reportedly upregulated by oxidative stress caused by H_2O_2 and γ-irradiation through 2 antioxidant response-like elements in thymic-sorted cells.[12] Interestingly, we observed that following the administration of high-fat diet, the increase in vanin-1 mRNA levels (seen as early as Day 1 after high-fat diet) preceded the onset of lipid accumulation, the hallmark of NAFLD (seen at 2 weeks after high-fat diet). Oleic acid, in particular, at concentrations of ≥0.01 mM was able to significantly upregulate vanin-1 mRNA expression *in vitro* when compared with control cultures (Fig. 4B); however, lipid droplets were not observed at oleic acid concentrations of 0.01 and 0.1 mM. Hence, it is possible that oxidative stress produced through beta oxidation of FFAs in the hepatocytes contributes to the induction of vanin-1 gene, and marks an early stage of NAFLD. The pantetheinase activity of

vanin-1 hydrolyzes pantetheine, an intermediate metabolite of coenzyme A, into pantothenic acid (vitamin B5) and cysteamine, a potent antioxidant.[26] Pantothenic acid, pantothenol, and other derivatives are known to protect cells and whole organs against peroxidative damage by increasing the content of cellular glutathione.[27] Therefore, the upregulation of vanin-1, leading to increased levels of cysteamine, may be an adaptive mechanism against hepatic oxidative stress induced by high-fat diet.

Previously, it has been reported that hepatic vanin-1 mRNA was upregulated in hepatic steatosis.[7–9] Further, PPARα is a major coordinator of fatty acid oxidation in the liver.[28] FFAs increase PPARα mRNA expression, and its activation regulates several key genes involved in fatty acid uptake and β-oxidation.[29,30] We have previously shown that a high-fat diet induces the mRNA expression of ADRP,[31] which is a reliable lipid droplet marker in fatty liver condition.[32] Furthermore, it has been reported that vanin-1 mRNA is upregulated by PPARα activation.[33–36] In the present study, we observed that PPARα, but not vanin-1, is upregulated by all types of fatty acids. Hence, it is appealing to speculate that in addition to PPARα, vanin-1 is regulated by another upstream factor, whose expression/activity is controlled by the uptake of FFAs, and which may play a role in lipid metabolism in the liver.

NAFLD is characterized by fatty infiltration of the liver in the absence of alcoholic consumption, and currently affects approximately 30% of the adults and 10% of the children in the United States. It ranges from simple steatosis to NASH, which can progress to end-stage liver disease.[24,25] The development of NAFLD is closely associated with obesity and type 2 diabetes.[6] Previous reports have suggested hyperinsulinemia and inappropriately high amounts of free fatty acids as a cause of hepatic steatosis.[37] However, a greater understanding of the pathophysiological changes is needed to develop more effective therapies for NAFLD.[6] We therefore believe that our mouse model for high-fat diet-induced hepatic steatosis would be useful to study the pathophysiology and regulatory factors involved in the progression of steatosis to NASH. Because NASH is thought to be a mitochondrial-dysfunction disease, which is caused by the overproduction of reactive oxygen species that in turn triggers lipid peroxidation,[38] it is quite likely that upregulation of vanin-1 plays a role in disease progression.

Our findings reveal that the upregulation of vanin-1 precedes lipid accumulation and is differentially mediated by various types of FFAs in the fatty liver model, presenting vanin-1 as a novel player in the pathogenesis of NAFLD. Further experiments using vanin-1 knockout mice would be necessary to establish the function of vanin-1 in the progression of NAFLD.

Acknowledgments

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Conflict of Interest

No potential conflicts of interest were disclosed.

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