Biochemical and Biophysical Research Communications 422 (2012) 22–27

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Ezetimibe reduces fatty acid quantity in liver and decreased inflammatory cell infiltration and improved NASH in medaka model

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ARTICLE INFO

Article history:
Received 17 April 2012
Available online 25 April 2012

Keywords:
NASH
Ezetimibe
Medaka
Inflammation

ABSTRACT

Purpose: We previously developed medaka non-alcoholic steatohepatitis (NASH) model. The model showed similar histology with human NASH so we analyzed the effect of drug using medaka NASH activity score (MNAS). In this study we analyzed the effect of ezetimibe, a small intestine cholesterol transporter inhibitor, on NASH.
Methods: Medaka NASH model showed steatohepatits with infiltration of D-PAS positive inflammatory cell. In this study we induced medaka NASH and compared the effect of ezetimibe on medaka NASH by HFD.
Results: As compared with the HFD group, ezetimibe reduced total cholesterol and triacyglycerol in the blood. But concerning with liver quantity of fatty acids in the liver were significantly decreased by ezetimibe. Genes related with fatty acid metabolism in liver was also decreased by ezetimibe administration. On histological observations of the liver, increases in the number of inflammatory cells and MNAS were inhibited. With this decrease of fatty acid in liver, medaka NASH was improved by ezetimibe.
Conclusion: Ezetimibe was clarified as a useful drug to improve NASH.

1. Introduction

Non-alcoholic steatohepatitis (NASH) is a disease concept first reported by Ludwig et al. in 1980 [1]. The disease state resembles alcoholic liver disease, even though alcohol consumption is not noted in amounts sufficient to cause liver damage. It progresses from simple fatty liver to steatohepatitis and cirrhosis, and finally to hepatocarcinogenesis [1]. The number of patients with NASH is currently increasing worldwide, with the most widely supported theory on the cause of NASH being the two-hit theory proposed by Day et al., in which fatty liver occurs first (first hit), followed by a transition to steatohepatitis (second hit) [2]. However, the specific onset mechanisms of NASH have not yet been adequately elucidated. The thinking to date is that the first hit of fatty liver occurs against a background of symptoms including hypertension, dyslipidemia and glucose intolerance, comprising so-called metabolic syndrome, which then progresses to NASH as a result of unknown factors [2]. NASH further progresses to cirrhosis, and finally to hepatocarcinogenesis.

There are currently no established effective treatments for NASH, and the mechanism of the progression from simple fatty liver to NASH has not been completely elucidated either. Mice, rats and other rodents have been widely used in basic studies on NASH, but small fish, such as zebrafish, have also come to be recognized as useful model animals.

Medaka are small fish similar to zebrafish that are native to many parts of Japan and Asia. In Japan, there are numerous pure line species used as animal models [3]. Furthermore, in comparison with rodents, medaka reproduce prolifically, mature rapidly and are small; thus, little space and cost are required for breeding. The medaka genome project has also been completed, and methods have been established for the generation of transgenic and knockout animals, thereby fulfilling the necessary conditions for animal models [4]. Past reports on models with liver fatty changes in small fish include zebrafish mutants (*foie gras* mutants) [5], mutant medaka identified via genetic screening by *N*-ethyl-*N*-nitrosourea (ENU) treatment (Kendama mutants) [6], and a zebrafish model with liver fat accumulation due to expression of HBx protein [7]. In these reports, however, the models were prepared using mutants or genetic manipulation, and as of this writing, the only model exhibiting a profile resembling human NASH through feeding wild-type medaka a high-fat diet is our previously reported medaka NASH model [8].

After developing this human-like medaka NASH model [8], we demonstrated that it presents a pathological condition similar to human NASH, and that n-3 polyunsaturated fatty acid is very important in NASH pathology [8]. We have also demonstrated that

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.04.087

telmisartan, which has angiotensin II type 1 receptor-inhibiting and PPAR-γ-stimulating actions, inhibits liver macrophage infiltration and inhibits the accumulation of liver fat, thereby improving the pathological condition of the medaka NASH model [9]. In the present study, we administered ezetimibe, a small intestine cholesterol transporter (Niemann–Pick C1-Like 1) inhibitor, to the medaka NASH model, and clarified the effects of ezetimibe in this model.

2. Materials and methods

2.1. Animals

Himedaka strain Cab (an orange-red variety of medaka, *Oryzias latipes*) aged 8 weeks were used for most experiments. Fish were maintained at a stock level of 10 fish per tank in tap water with aeration. All 10 fish in a given tank received a daily ration of 200 mg of the diet prescribed for that group, and this was consumed completely within 14 h. All fish were maintained in accordance with the Animal Care Guidelines of Yamaguchi University. Medaka were divided into three groups: those fed HFD only (HFD group), control normal diet and those given HFD containing ezetimibe (HFD + ezetimibe group).

2.2. Diet

The proportions of protein, fat and carbohydrate, as well as the fatty acid compositions, of the control and HFD that were used in this study were as previously reported [8]. Ezetimibe was pulverized into a powder and mixed with HFD to give a dose of 0.01 mg per day, and was administered.

2.3. Histology

Euthanized fish were slit open from the anal vent to the gills, and the entire body was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (Muto, Tokyo, Japan). The liver was dissected, dehydrated in alcohol, and embedded in paraffin in accordance with standard procedures. Serial sections were cut and stained with hematoxylin and eosin (H&E). Liver macrophages were assessed by diastase-pas staining (DPAS staining). Five × 400-power fields were photographed in random locations on individual liver specimens stained with DPAS, and the number of DPAS-positive cells in each field was counted. In addition, a medaka NAFLD activity score (MNAS) [9] was determined based on human NAFLD activity score [10], and the tissue of individual livers was scored based on this. Trends in NASH severity were then quantitatively evaluated.

2.4. Blood analysis

Blood samples were analyzed as described [9]. Cholesterol and TG profiles in total lipoproteins were analyzed using a dual-detection HPLC system with two tandem-connected TSKgel Lipopropak XL columns (300 × 7.8 mm; Tosoh, Japan) by Skylight Biotech (Akita, Japan). The results were shown in Table 1A and B.

2.5. Measurement of triacylglycerol content and fatty acid in liver tissue

At 8 weeks after the start of the experiment, triacylglycerol content and fatty acid fractionation in liver tissue were measured in individual fish. Triacylglycerol in liver tissue was extracted using the method described by Folch et al. [11]. Fatty acids were methylated with boron trifluoride and methanol. Methylated fatty acids were analyzed as described [9].

Table 1
Blood lipid test results for the Control group, HFD 8 weeks group, and HFD + ezetimibe 8 weeks group (A and B). In each group, serum from 20 animals was collected and measurements were performed.

	Total	CM	VLDL	LDL	HDL
A					
Serum cholesterol concentration (mg/dL)					
Control	223.3	1.6	78.8	28.6	114.3
HFD 8 weeks	245.5	64.3	125.7	25.7	29.8
HFD + ezetimibe 8 weeks	132.0	21.7	62.3	13.8	34.1
B					
Serum triglyceride concentration (mg/dL)					
Control	509.6	6.6	248.5	45.2	209.4
HFD 8 weeks	2433.4	572.5	1187.7	265.2	408.0
HFD + ezetimibe 8 weeks	1202.5	202.4	553.2	94.1	352.8

2.6. Real-time RT-PCR analysis

Quantitative real-time RT-PCR was performed as described [12]. Primer sequences are listed in Supplementary information (Table S1).

2.7. Statistical analyses

Numerical data are expressed as means ± S.D. Student's *t*-test was performed in order to assess the statistical significance among the groups of medaka. *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Changes in morphology, body weight and body mass index

On gross observations, abdominal distention (Fig. 1A) and whitish liver color (Fig. 1B) were seen in medaka of the HFD group. In the group given ezetimibe, abdominal distention improved (Fig. 1C) and liver color changed from white to dark red (Fig. 1D)(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.). Body length and body weight of each medaka were measured at the start of the experiment and after 4, 8, and 12 weeks on the respective diets. Body mass index (BMI) was calculated based on these measurements. In the HFD and HFD + ezetimibe groups, no differences were seen in changes in body weight or BMI throughout the entire experiment (Fig. 1E and F).

3.2. Ezetimibe ameliorated dyslipidemia in serum medaka NASH model

At 8 weeks after the start of the experiment, blood lipid tests were performed in the HFD and HFD + ezetimibe groups. The HFD group had higher total cholesterol and triacylglycerol levels than the Control group (total cholesterol: Control group, 223.3 mg/dl, HFD 8 weeks group, 245.5 mg/dl; triacylglycerols: Control group, 509.6 mg/dl, HFD 8 weeks group, 2433.4 mg/dl). Meanwhile, the HFD + ezetimibe group had lower total cholesterol and triacylglycerol levels than the HFD group (total cholesterol: HFD + ezetimibe 8 weeks group, 132.0 mg/dl; triacylglycerols: HFD + ezetimibe 8 weeks group, 1202.5 mg/dl) (Table 1). These results suggested that ezetimibe decreased high cholesteral and triacylglerol induced by HFD in serum.

3.3. Ezetimibe inhibited fatty acid accumulation in the liver in medaka NASH model

Next, the amount of fatty acid in the liver was measured, and at 8 weeks after the start of the experiment, the HFD group had

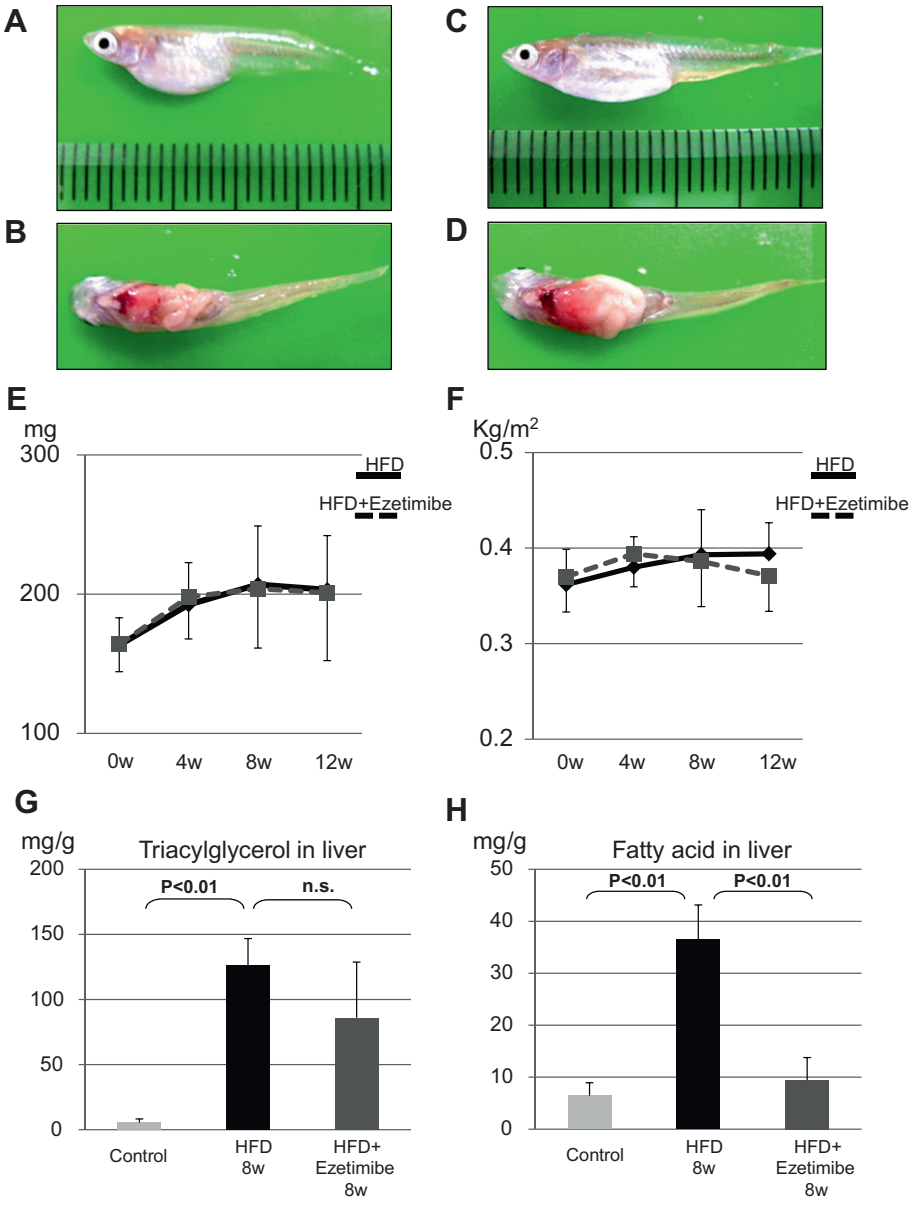

Fig. 1. Change in morphology after HFD for 12 weeks. Abdominal distension was observed (A). Liver showed a whitish color (B). Abdominal distention was reduced (C). Liver showed a brown color (D). Changes in body weight from the start to study completion in the HFD group and HFD + ezetimibe group (E). Change in body mass index from the start to study completion between the HFD group and HFD + ezetimibe group (F). Liver fat contents in the Control group, HFD 8 weeks group, and HFD + ezetimibe 8 weeks group (G). Liver fatty acid contents in the liver in the Control group, HFD 8 weeks group, and HFD + ezetimibe 8 weeks group (H). Data are means ± SD. n.s., no significant difference. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

markedly higher triacylglycerol and fatty acid levels in the liver than the Control group (triacylglycerols: Control group, 5.5 ± 2.8 mg/g; HFD 8 weeks group, 126.6 ± 20.3 mg/g ($p < 0.01$); fatty acid: Control group, 6.5 ± 2.5 mg/g, HFD 8 weeks group, 36.5 ± 6.6 mg/g ($p < 0.01$), and the HFD + ezetimibe group had lower levels of liver fatty acid than the HFD group (liver fatty acid: HFD 8 weeks group, 36.5 ± 6.6 mg/g, HFD + ezetimibe 8 weeks group, 9.4 ± 4.3 mg/g ($p < 0.01$) (Fig. 1G and H).

3.4. Ezetimibe improved MNAS with decrease of infiltration of inflammatory cells in medaka NASH model

The HFD group had higher inflammatory cell infiltration in the liver at 8 and 12 weeks than the Control group (Control group, 0.6 ± 1.1/HPF, HFD 8 weeks group, 3.6 ± 3.0/HPF ($p < 0.01$), HFD 12 weeks group 7.9 ± 3.9/HPF ($p < 0.01$). At 4 and 12 weeks, the HFD + ezetimibe group showed inhibition of inflammatory cell infiltration in the liver, as compared to the HFD group (HFD 4 weeks group, 0.9 ± 1.6/HPF, HFD + ezetimibe 4 weeks group, 0.2 ± 0.6/HPF ($p < 0.05$); HFD 12 weeks group, 7.9 ± 3.9/HPF, HFD + ezetimibe 12 weeks group, 1.5 ± 2.2/HPF ($p < 0.01$) (Figs. 2, 3A).

3.5. Ezetimibe slowed progression of medaka NAFLD activity scores in medaka NASH model

The HFD group had significantly higher medaka NAFLD activity scores (MNAS) than the Control group at 8 and 12 weeks (Control group, 0.4 ± 0.5, HFD 8 weeks group, 4.0 ± 2.2 ($p < 0.05$), HFD 12 weeks group, 5.8 ± 1.5 ($p < 0.01$), while the HFD + ezetimibe group had significantly lower MNAS than the HFD group at 12 weeks (HFD 12 weeks group 5.8 ± 1.5, HFD + ezetimibe 12 weeks group, 2.0 ± 1.0 ($p < 0.05$) (Fig. 3B).

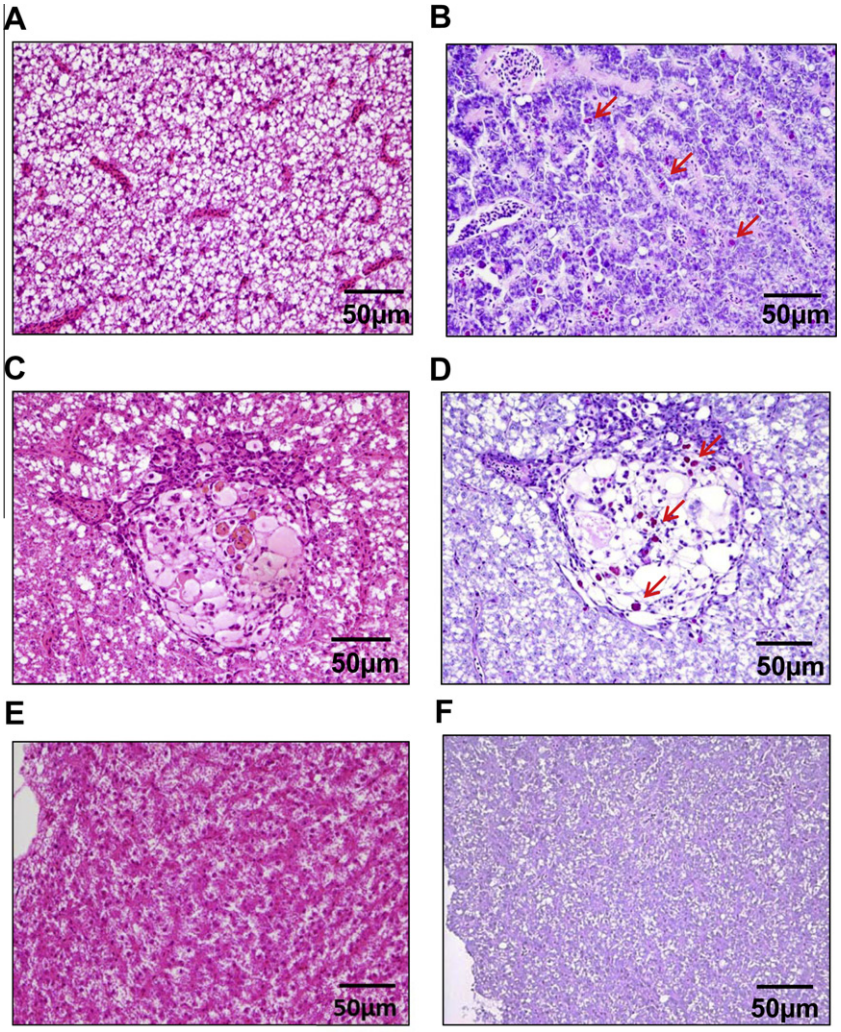

Fig. 2. Fat deposition in the liver at 8 weeks in the HFD. HE staining (A); DPAS-positive cells in the liver at 8 weeks in HFD (Arrow indicated). D-PAS staining (B); balloon-like changes in liver cells (C); DPAS-positive cells in this state (Arrow indicated) (D); liver after 8 weeks in the HFD + ezetimibe group (E); decreased fat deposition in the liver at 8 weeks (F). Measurements were performed in each ×400-power field. Data are means ± SD. n.s., no significant difference.

3.6. Changes in expression of lipid-related genes in liver

Investigation of lipid-related gene expression in liver revealed markedly decreased expression of PPAR alpha, AC03, MCAD, ACC1 and APoB in the group administered ezetimibe (Fig. 3C–I). These results indicated that gene related with fatty acid synthesis and oxidation were decreased.

4. Discussion

We previously developed and reported a medaka NASH model as an effective new model for drug screening for NASH [8]. The present study involved evaluation and analysis using ezetimibe in the medaka NASH model. Ezetimibe is a novel sterol absorption inhibitor that blocks Niemann–Pick C1-Like 1 (NPC1L1) – mediated cholesterol enterocytes [13]. It has already been reported, among other things, in animal experiments that ezetimibe improves dietary obesity [14,15], improves liver fatty change [14–17], and improves insulin resistance [16]. Using medaka NASH model, we also reported that telmisartan, which has angiotensin II type 1 receptor-inhibiting and PPAR-γ-stimulating actions, inhibits liver macrophage infiltration and inhibits the accumulation of liver fat, thereby improving the pathological condition of the medaka NASH model [9].

The effect of ezetimibe is quite different from telmisartan, In this study, however ezetimibe improved the abdominal distention caused by the high-fat diet and fat deposition in the liver (Fig. 1A–D), but ezetimibe did not produce major changes in BMI or body weight (Fig. 1E and F). However, ezetimibe decreased the amount of triacylglycerol and fatty acid in serum (Table 1A and B). Moreover the amount of fatty acid in the liver was significantly suppressed (Fig. 1G and H). When diagnosing the histopathological picture of NASH in humans, NAFLD activity score (NAS), which numerically quantifies the three factors of steatosis, inflammation and ballooning, is widely used [10]. We created the medaka NAFLD activity score (MNAS), and showed in analyses conducted with our medaka NASH model that the score increased as the pathological condition progressed with time. In recent studies, the increase in the number of macrophages infiltrating adipose tissue is reported to be related to obesity [18]. A comparative analysis of the HFD group and Control group looking only at inflammatory cells showed that, similar to MNAS, the number of inflammatory cells had increased after 8 and 12 weeks of HFD administration. These results indicated that inflammatory cells serve an important role in the progression of the NASH condition, (Fig. 2A–D), but ezetimibe group we could not find D-PAS positive cell (Fig. 2E and F). These results indicated that ezetimibe significantly inhibited the

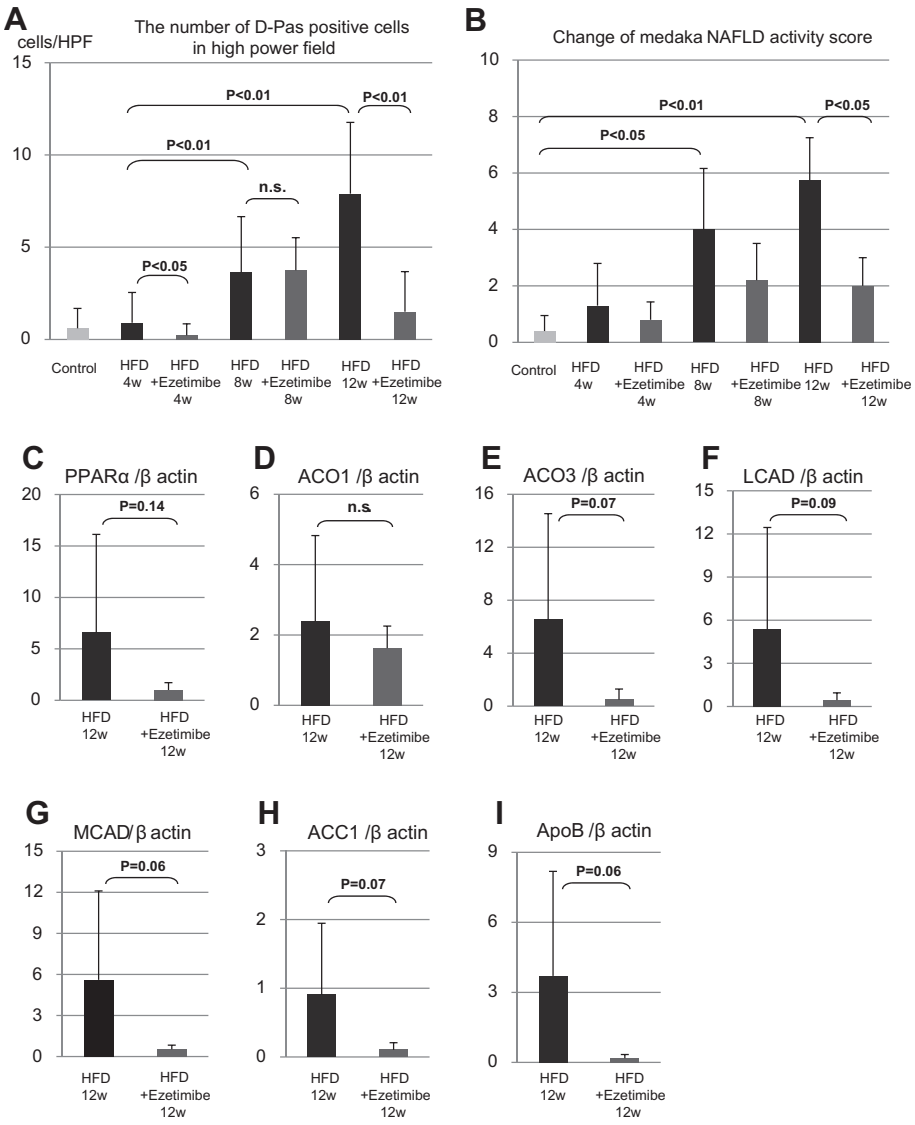

Fig. 3. Changes in DPAS-positive cells (A). Changes in MNAS (B). Trends in liver medaka NAFLD activity score in the HFD group and HFD + ezetimibe group. 4, 8 and 12 weeks data was presented. Scoring and quantification were performed for individual livers. Changes in gene expression of PPAR alpha (C), ACO1 (D), ACO3 (E), LCAD (F), MCAD (G), ACC1 (H) and ApoB (I) in the liver in the HFD group and HFD + ezetimibe group at 12 weeks.. Data are means ± SD. n.s., no significant difference.

infiltration of inflammatory cells in a medaka liver and MNAS was improved (Fig. 3A and B).

In medaka model, as shown in Fig. 3C–I, the amount of gene expression related to fat metabolism in liver tissue was decreased. Fatty acid synthesis and oxidation was decreased (Fig. 1H). Thus, ezetimibe administration was shown to decrease lipid synthesis activity in the liver. Numerous reports have ruled out the possibility that ezetimibe inhibits fatty acid absorption, although it has been reported that the expression of fatty acid transport protein 4 is decreased and fatty acid absorption is inhibited when NPC1L1 is knocked out in mice [19]. In addition, there have been no reports clarifying how fatty acids are absorbed in the small intestine of medaka. In this model, ezetimibe decreased the amount of triacylglycerol and fatty acid in serum and decreased the fatty acid in liver. This ezetimibe effect was quite effective for improvement of MNAS.

Moreover, according to a reported gene expression analysis in the liver of human NAFLD patients, the expression of genes related to fatty acid cleavage and fatty acid binding, lipolysis, macrophage migration and inflammation are elevated in NAFLD patients [20]. The infiltration of inflammatory cells, such as monocytes and macrophages, plays an important role in the progression of metabolic syndrome, and inflammatory cells are conjectured to serve important roles even in NASH, which is thought to be an expression of metabolic syndrome in the liver [18,21]. The present results confirmed an obvious decrease in inflammatory cells together with the decrease in the amount of fatty acid in the NASH liver. These results also showed that ezetimibe had an effect on anti-inflammation associated with metabolic syndrome.

In the present investigation, the results indicated that in the group administered ezetimibe, fat deposition in the liver was improved, and this was accompanied by an improvement in inflammation. Therefore, ezetimibe may have the potential to improve the inflammatory state that occurs in the body in association with metabolic syndrome.

In conclusion, we were able to observe the pathological changes resembling human NASH by administering a HFD to medaka. In this model, ezetimibe was effective in improving the pathological condition.

Acknowledgments

This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science

(22659148, 23659398), the Japan Science and Technology Agency, and the Ministry of Health, Labour and Welfare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.087.

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