Deletion of TRPC3 or TRPC6 Fails to Attenuate the Formation of Inflammation and Fibrosis in Non-alcoholic Steatohepatitis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a liver disorder that resembles alcoholic liver disease despite no apparent drinking history. 1 Obesity is the most important factor involved in the development of NAFLD. 2,3 NAFLD is a disease that includes a non-alcoholic fatty liver (NAFL) and Non-alcoholic steatohepatitis (NASH). The accumulation of triglycerides leads to the development of NAFL. NASH is an advanced form of NAFL in which there is an infiltration of inflammatory cells and fibrosis in liver tissue similar to alcoholic steatohepatitis. 4,5 With regard to the progression of inflammatory cell infiltration and fibrosis, it is basically believed that inflammatory cell infiltration occurs first, followed by fibrosis. In epidemiology, dyslipidemia, hypertension, fasting hyperglycemia, and metabolic syndrome is associated with NASH. 3,4

Transient receptor potential (TRP) channels have been known as multimodal cation channels and associated with many diseases such as cardiovascular disease. 6 Especially canonical TRP channel subfamily members 3 (TRPC3) and 6 (TRPC6), have been reported to be involved in the development of pathological fibrosis caused by neurohumoral factors and mechanical stress in the heart. 7 In TRPC3-deficient mice, cardiac fibrosis is diminished in response to pressure overload. 7 TRPC3/C6 deletion ameliorates cardiac hypertrophy and fibrosis induced by pressure overload. 8,9 Additionally, selective inhibition of TRPC6 improves left heart function and fibrosis in mice that are subjected to sustained pressure overload. 8 TRPC3 forms complexes with reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) 2 and increases reactive oxygen species (ROS). ROS are one of the major causes in NASH. 10,11 It has also been reported that cations including calcium are significantly associated with the pathogenesis of NASH. 12 However, it is not clear whether TRPC3 and TRPC6 are involved in progression of NASH. The aim of this study is to clarify the role of TRPC3 and TRPC6 in the formation of NASH and to show whether these channels are therapeutic targets. In this study, we examined that the role of TRPC3 and TRPC6 in NASH using the choline-deficient, t-amino acid-defined, high-fat diet (CDAHFD) model and genetically modified mice of TRPC3 and TRPC6.

MATERIALS AND METHODS

Animals All procedures used in this study were approved by the ethic committees at the Animal Care and Use Committee, Kyushu University. We produced C57BL/6 strain mice with systemic knockout (KO) of TRPC3 or TRPC6 (8–11 weeks old, male) by backcrossing 129Sv strain TRPC-deficient mice kindly provided by Dr. Birnbaumer (NIEHS, U.S.A.) onto C57BL/6 backgrounder mice. Animals were maintained under a 12 h/12 h light/dark cycle.

Test Diets Male mice were fed with a Choline deficient
Fig. 1. Increase in TRPC3 mRNA Expression Level in CDAHFD-Fed Mouse Liver

Quantitative real-time PCR of TRPC3 and TRPC6 genes of the livers in C57BL/6J mice fed with standard diet (Control) or choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD) for 6 weeks. TRPC3 (A) and TRPC6 (B). Expression levels of mRNA were normalized to 18s rRNA. Data are shown as the mean ± standard error of the mean (S.E.M.) (n = 5 in each group). *p < 0.05, Student’s t-test.

Fig. 2. Effects of TRPC3 or TRPC6 Deletion on CDAHFD-Induced Changes in Body and Tissue Weights

WT, TRPC3 KO and TRPC6 KO were fed with CDAHFD for 6 weeks. (A) Body weight (B–F) Tissue weights of liver (B), spleen (C), BAT (D) and WAT (E). Data are shown as the mean ± S.E.M. (n = 5 in each group). *p < 0.05, **p < 0.01, one-way ANOVA followed Tukey’s comparison test.
L-amino acid-defined high fat diet (CDAHFD diet, Cat# A06071302, Research Diets Inc., New Brunswick, NJ, U.S.A.) or control diet (Cat# A06071314, Research Diets Inc., New Brunswick, NJ, U.S.A.) for 6 weeks. After 6 weeks, all mice were euthanized under anesthesia with isoflurane. The liver, spleen, brown adipose tissue (BAT), and white adipose tissue (WAT) were taken and all tissue weights were measured. Blood samples were collected from the caudal vena cava and centrifuged at 10,000 × g for 10 min.

**Serum Biochemical Analysis** Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), high density lipoprotein cholesterol (HDLC), total cholesterol (TC) and triglyceride (TG) were measured using an Automatic Biochemistry Analyzer (Fuji Dry-Chem NX5000; FUJIFILM Medical, Tokyo, Japan).

**Liver Histology** The liver was fixed with 10% neutral buffered formalin and embedded in paraffin. Hematoxylin and eosin (H&E) staining was performed as previously described. Empty envelopes were quantified using ImageJ and evaluated as steatosis.

**RNA Isolation and Quantitative Real-Time RT PCR** Total RNA was extracted and complementary DNA was synthesized as previously described. Quantitative real-time PCR was performed as previously described. Primer sequences used are summarized in supplementary Table 1. In order to normalize cDNA levels, 18s ribosomal RNA (rRNA) expression was used as an endogenous control.

**Statistics** We performed statistical analysis by using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, U.S.A.). All results were expressed as mean ± standard error of the mean (S.E.M.) from at least 5 independent experiments and were considered significant when p < 0.05. Statistical comparisons were determined using two-tailed Student’s t-test (for two groups) or using one-way ANOVA with Tukey’s post
**RESULTS**

**TRPC3 Increased in NASH Model** To investigate whether the expression of TRPC3 and TRPC6 are changed in NASH model, we first examined the mRNA expression of TRPC3 and TRPC6 in liver (Figs. 1A, B). Expression level of TRPC3 was increased in CDAHFD fed mice compared with standard (control) diet (Fig. 1A).

**Body and Tissue Weights** Next, we investigated the role of TRPC3 and TRPC6 in a diet-induced NASH model using TRPC3 KO mice and TRPC6 KO mice. WT mice fed with CDAHFD diet for 6 weeks lost body weight. The body weight of TRPC3 KO mice was decreased in the same manner as WT mice, but the body weight of TRPC6 KO mice was more slowly decreased than WT mice. The body weight loss of TRPC6 KO mice fed with CDAHFD was significantly suppressed in comparison to WT mice fed with CDAHFD (Fig. 2A). We confirmed that the food intake in TRPC6 KO mice was similar to that in WT and TRPC3 KO mice (Fig. 2B). WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD diet had significantly increased liver and spleen weight in comparison to WT mice fed with CDAHFD (Fig. 2A). We confirmed that the food intake in TRPC6 KO mice was similar to that in WT and TRPC3 KO mice (Fig. 2B). WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD diet had significantly increased liver and spleen weight than those fed with control diet (control) (Figs. 2C–F). Liver weights in TRPC3 KO mice fed with CDAHFD were reduced in comparison to WT mice fed with CDAHFD. In contrast, spleen, BAT, and WAT weights were similar in WT, TRPC3 KO and TRPC6 KO mice (Figs. 2C–F).

**Serum Levels of AST, ALT, HDLC, TCHO and TG** To evaluate liver damage, we measured serum levels of liver-damaging enzymes such as ALT and AST. Serum levels of ALT and AST were significantly increased in WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD diet in comparison to WT mice fed with control diet. However, ALT and AST were similar in WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD (Figs. 3A, B). We then measured lipid levels in the serum. HDLC and TCHO were significantly decreased in WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD diet in comparison to WT mice fed with control diet. TRPC6 KO mice compared to WT mice had significantly increased HDL cholesterol in CDAHFD diet (Fig. 3C). TCHO was similar in WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD (Fig. 3D). TG was similar in WT mice fed with control diet or CDAHFD, TRPC3 KO and TRPC6 KO mice fed with CDAHFD (Fig. 3E).

**Liver Histology** Histopathology data showed that WT mice fed with CDAHFD diet caused predominantly middle droplet steatosis and induced infiltration of inflammatory cells as stained with H&E (Fig. 4A). Steatosis was significantly increased in WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD diet in comparison to WT mice fed with control diet. Steatosis was similar in WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD (Fig. 4B). Infiltration of inflammatory cells was similar in WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD (Fig. 4A).

**Factors Involved in Inflammation and Fibrosis** C–C motif chemokine 2 (CCL2) and tumor necrosis factor-α (TNFα) were measured as factors involved in liver inflammation. CCL2 was significantly increased in WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD diet in comparison to WT mice fed with control diet. CCL2 and TNFα were similar in WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD (Figs. 5A, B). TIMP metalloproteinase inhibitor 1 (TIMP-1) and Collagen Type I Alpha 1 Chain (COL1A1) were measured as a factor involved in fibrosis. Both factors were significantly increased in WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD diet in comparison to WT mice fed with control diet. Both TIMP-1 and COL1A1 were similar in
DISCUSSION

In this study, we found that expression of TRPC3 is increased in mice fed with CDAHFD (Fig. 1A). In addition, liver weights in TRPC3 KO mice fed with CDAHFD were reduced compared with in WT mice fed with CDAHFD (Fig. 2C). On the other hand, although the expression of TRPC6 was not increased in mice fed with CDAHFD, TRPC6 deficiency suppressed the decrease in body weight and HDLC (Figs. 2C, 3C). These results suggest that the physical status in TRPC6 KO mice fed with CDAHFD was improved in comparison to WT mice fed with CDAHFD. However, liver steatosis, inflammation and fibrosis were not significantly different among and WT, TRPC3 KO and TRPC6 KO mice fed with CDAHFD (Figs. 4, 5). Both TRPC3 and TRPC6 are expressed in various tissues other than the liver. TRPC3 is highly expressed in the heart and blood vessels, and acts as a channel and scaffold protein. We found that TRPC3 is involved in liver weight increase induced by CDAHFD independent of inflammation and fibrosis. Thus, TRPC3 deficiency in tissue(s) other than liver may indirectly contribute to liver weight increase induced by CDAHFD feeding. In addition, TRPC6 is also expressed in various tissues such as kidney and lung, and it might be possible that deficient of TRPC6 in these tissues indirectly led to the improvement of body weight loss. Food intakes were not significantly different among WT, TRPC3 KO and TRPC6 KO mice, but food intake of TRPC6 KO tended to be greater than WT and TRPC3 KO mice (Fig. 2B). A recent paper has suggested that TRPC6 deficiency causes obesity and metabolic dysfunction with increasing food intake in mice. We could not find any obese phenotype or metabolic dysfunction in TRPC6 KO mice, but TRPC6 might have some influence on appetite.

TRPC3 and TRPC6 are known to be activated by diacylglycerol downstream of the muscarinic receptor. The muscarinic receptor has been reported to be associated with liver fibrosis. However, we found that gene deletion of TRPC3 or TRPC6 alone cannot significantly suppress the increased expressions of inflammation and fibrosis markers in NASH model. Other TRPCs might compensate for the functional deficiency of TRPC3 or TRPC6. In fact, it has been reported that the deletion of TRPC3 or TRPC6 alone is not protective against pressure overload-induced cardiac fibrosis, whereas their combined deletion is protective. Therefore, deletion of both TRPC3 and TRPC6 genes might suppress inflammation and fibrosis in the NASH model.

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Conflict of Interest The authors declare no conflict of interest.
**Supplementary Materials** The online version of this article contains supplementary materials.

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