JDI Journal of Diabetes Investigation Open access
Official Journal of the Asian Association for the Study of Diabetes
ORIGINAL ARTICLE

Nicotinic alpha-7 acetylcholine receptor deficiency exacerbates hepatic inflammation and fibrosis in a mouse model of non-alcoholic steatohepatitis

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Keywords
Fibrosis, Inflammation, Non-alcoholic steatohepatitis

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J Diabetes Investig 2019; 10: 659–666

doi: 10.1111/jdi.12964

ABSTRACT

Aims/Introduction: Non-alcoholic steatohepatitis (NASH), which occurs in association with insulin resistance and hepatic fat accumulation, is characterized by chronic liver injury and fibrosis. NASH onset and progression is closely related to hepatic inflammation, which is partly regulated by the vagus nerve through the α7 nicotinic acetylcholine receptor (α7nAchR). Hepatic α7nAchR action is impeded in obesity and insulin resistance. In the present study, using α7nAchR knockout (α7KO) mice, we elucidated the effect of α7nAchR deficiency on NASH-related inflammation and fibrosis.

Materials and Methods: α7KO mice were fed an atherogenic high-fat diet (AD) for 32 weeks or methionine/choline-deficient diet (MCD) for 6 weeks, both of which induce NASH. Mice were then examined for the degree of NASH-related inflammation and fibrosis by hepatic gene expression analysis and Sirius red histological staining.

Results: Hepatic triglyceride accumulation and elevated plasma transaminase levels were observed in both AD and MCD mice, but the plasma transaminase level increase was higher in α7KO mice than in control mice. α7KO mice fed an AD showed significant upregulation of the *Col1a1* gene encoding alpha-1 type I collagen, which is involved in liver fibrosis, and the *Ccl2* gene encoding C-C motif chemokine ligand 2, a pro-inflammatory chemokine; α7KO mice fed an MCD had significant upregulation of the *Col1a1* gene and the *Tnf* gene, an inflammatory cytokine. Histological analysis showed that AD and MCD exacerbated liver fibrosis in α7KO mice.

Conclusions: The results of this study suggest that α7nAchR deficiency exacerbates hepatic inflammation and fibrosis in a diet-induced mouse model of NASH.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), which occurs in association with insulin resistance and is characterized by fat accumulation in the liver, is a disease spectrum that begins with simple fatty liver and progresses to non-alcoholic steatohepatitis (NASH) accompanied by cell injury/fibrosis and eventually cirrhosis[1]. In the spectrum of NAFLD, the part of the liver with simple steatosis progresses to NASH/cirrhosis, with inflammation playing an important role in the process. Indeed, hepatic inflammation is upregulated in NASH, with increases in the number of hepatic macrophages and the expression of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα), interleukin-6 and C-C motif chemokine ligand 2 (CCL2)[2,3]. Macrophages undergo polarized activation to pro-inflammatory M1 or anti-inflammatory M2 states, which involve the expression of CD11c or CD206 and CD163, respectively[4]. In NASH, the hepatic macrophage polarization shifts to the M1 state[5,6]. Furthermore, NASH is exacerbated by inflammatory inducers, such as pro-inflammatory cytokines, bacterial

Received 30 July 2018; revised 4 October 2018; accepted 25 October 2018

cell components derived from intestinal microbiota and saturated fatty acids[1,7]. In contrast, anti-inflammatory factors, such as adiponectin and unsaturated fatty acids, suppress the progression to NASH, suggesting their potential as candidate drugs for the prevention and treatment of NASH[8,9].

The vagus nerve regulates inflammation through the action of acetylcholine[10]. In a mouse study, electrical stimulation of the vagus nerve decreased the blood levels of TNFα and interleukin-6[11]. We have also shown the upregulation of hepatic inflammatory responses, such as increased interleukin-6 gene expression in the liver after vagotomy[12]. The vagus nerve regulates inflammatory responses in macrophages and Kupffer cells through α7 nicotinic acetylcholine receptor (α7nAchR)[12,13]. Indeed, α7nAchR-knockout (α7KO) mice have high levels of pro-inflammatory cytokines in the blood during endotoxemia, and in these mice, electrical stimulation of the vagus nerve fails to decrease plasma TNFα levels[13]. The vagal regulation of inflammation through α7nAchR is closely associated with the regulation of glucose metabolism. α7KO mice develop insulin resistance when fed a high-fat diet (HFD) that induces obesity[14]. We have also shown that the vagal α7nAchR action plays an important role in the brain-mediated regulation of hepatic glucose production[12]. The brain detects elevated plasma insulin and amino acid levels, resulting in the decrease in hepatic glucose production through the vagal regulation of inflammation through α7nAchR[12,15,16].

Along with the development of insulin resistance, which is an inducer of NASH, vagal fluctuation disappears, which is induced in response to changes in nutrient signals, such as plasma insulin and amino acid levels, and the action of the vagus nerve continues to weaken in the liver[12]. Given that vagotomy induces a hepatic inflammatory response, impaired vagal action through α7nAchR as a result of insulin resistance might be involved in the aggravation of NASH through the exacerbation of hepatic inflammation. However, the role of vagal α7nAchR action in the onset and exacerbation of NASH remains to be fully elucidated.

In the present study, α7KO mice were fed an atherogenic high-fat diet (AD) or methionine/choline-deficient diet (MCD), both of which induce NASH, and the effect of vagal α7nAchR impairment on the exacerbation of NASH-related inflammation and fibrosis was investigated. AD induces hepatic inflammation and fibrosis in addition to mild obesity and insulin resistance[2,17], whereas MCD induces a NASH-like pathology accompanied by hepatic inflammation and fibrosis, but not insulin resistance, by impairing the secretion of very low-density lipoprotein (VLDL) for hepatic release of triglycerides, and thus triggering their accumulation in the liver[2,17,18]. These diet-induced NASH animal models have shown that α7nAchR deficiency exacerbates NASH-related inflammation and fibrosis.

METHODS

Animals

All animal experiments were approved by the Animal Ethics Committee of Kanazawa University (approval number AP-132743), carried out according to the Animal Ethics Committee guidelines for the care and use of laboratory animals at Kanazawa University, and carried out following the national guidelines and the relevant national laws on the protection of animals. C57BL/6J Slc mice were purchased from Japan SLC (Shizuoka, Japan) and α7KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), and maintained in a temperature-controlled environment with a 12-h light/dark cycle and free access to food and water under specific pathogen-free conditions in the Institute for Experimental Animals of Kanazawa University. Male α7KO mice were obtained by mating heterogeneous α7KO mice, and wild-type littermates of α7KO mice were used as controls. Sample sizes are stated in the figure legends.

Diets and sample collection

Mice were fed an AD (D06061403; Research Diet, New Brunswick, NJ, USA) or MCD (A02082002B; Research Diet) for 32 or 6 weeks from 7 weeks-of-age, respectively. The AD provides 61% of energy from fat and 1.3 g cholesterol/100 g diet[19], and the MCD provides 21.2% of energy from fat, but lacks methionine and choline. At the end of the experimental period, plasma and tissue samples were collected from animals in the ad libitum-fed state and stored at −80°C.

Biochemical and histological analyses

Blood glucose levels were measured using a GLUCOCARD G+ Meter (Arkray, Kyoto, Japan). Plasma insulin concentrations were determined using a mouse insulin enzyme-linked immunosorbent assay kit (Wako, Saitama, Japan). Plasma aspartate aminotransferase/alanine aminotransferase (AST/ALT) levels were measured using the Transaminase CII-Test-Wako kit (Wako). Liver triglyceride concentrations were measured by using the TG E-Test-Wako (Wako), as described previously[20]. Liver tissues were fixed in 4% paraformaldehyde/phosphate-buffered saline (Wako), and the sections were stained with Sirius red.

Quantitative polymerase chain reaction

Quantitative polymerase chain reaction was carried out using the SYBR Select Master Mix kit (Thermo Fisher Scientific, Waltham, MA, USA), as described previously[21]. Quantitative polymerase chain reaction results were analyzed using the *Rplp0* gene as an internal control and plotted in arbitrary units as the mean ± standard error. Primer sequences for *Rplp0*, *Srebf1*, *Fasn*, *Scd1*, *Ppara*, *Cpt1a*, *Il6*, *Tnf*, *Ccl2*, *Acta2*, *Col1a1* and *Tgfb1* are as described previously[19,22,23]. Primer sequences for *Mttp*, *Itgax*, *Mrc1* and *Cd163* are described in Table S1.

Statistical analysis

Data are represented as the mean ± standard error. Statistical analysis was carried out using Student's *t*-test and one-way ANOVA followed by post-hoc tests, and differences were considered significant at $P < 0.05$.

RESULTS

Animals fed an AD are reported to develop NASH accompanied by insulin resistance, hepatic inflammation and liver fibrosis[2,17]. In the present study, AD induced a significant increase in body weight, blood glucose levels, plasma insulin and ALT levels, and hepatic triglyceride content (Figure 1a–e). We therefore investigated the effect of α7nAchR deficiency on insulin resistance and liver injury induced by the AD. α7KO mice fed an AD had significantly higher plasma ALT levels and, although insignificant, the levels of plasma AST were also increased (Figure 1d). However, body weights, and blood glucose and plasma insulin levels did not differ significantly between α7KO and control mice (Figure 1a–c). Hepatic triglyceride content was significantly increased in α7KO mice fed an AD (Figure 1e). Hepatic gene expression of *Srebf1*, a master regulator of hepatic lipogenesis, was higher in α7KO mice fed an AD than in their control (Figure S1a). However, there were no differences in the hepatic expression of lipogenic enzyme genes (*Fasn* and *Scd1*), lipid oxidation enzyme genes (*Ppara* and *Cpt1a*) or a VLDL secretion-associated gene (*Mttp*) between α7KO mice and their control (Figure S1a,b).

We also investigated the effect of α7nAchR deficiency on NASH-related inflammation and fibrosis. Hepatic gene expression analysis showed significant messenger ribonucleic acid upregulation of the pro-inflammatory mediators *Tnf*, *Il6* and *Ccl2* in mice fed an AD (Figure 2a). The expression of the *Ccl2* gene, but not the *Tnf* and *Il6* genes, was significantly higher in α7KO mice than in control mice (Figure 2a). There were no differences in the hepatic expression of the *Itgax*, encoding the M1 marker CD11c, *Mrc1*, encoding the M2 marker CD206, and *CD163* genes between the groups (Figure 1c). Mice fed an AD showed significant upregulation of the *Acta2* and *Col1a1* genes, which are associated with fibrosis, and a tendency toward an increase in the *Tgfb1* gene, another fibrosis-related gene (Figure 2b). In α7KO mice, expression of the *Col1a1* gene, but not the *Acta2* and *Tgfb1* genes, was significantly higher than in control mice (Figure 2b). Histological analysis with Sirius red, which stains collagen fibers, clearly showed the exacerbation of liver fibrosis in α7KO mice compared with control mice (Figure 3).

Next, we investigated the effect of α7nAchR deficiency on MCD-induced NASH, and found no significant difference in body weight and blood glucose and plasma insulin levels between control and α7KO mice (Figure 4a–c). In α7KO mice fed an MCD, plasma AST levels were significantly increased, whereas plasma ALT levels tended to increase (Figure 4d). Although increased, hepatic triglyceride content did not significantly differ between control and A7KO mice (Figure 4e). Hepatic gene expression levels of lipogenesis, lipid oxidation and VLDL secretion did not differ between control and α7KO mice (Figure S2a,b). The expression levels of *Tnf*, *Ccl2*, *Acta2*, *Col1a1*, *Tgfb1* and *Itgax* genes were upregulated in the liver of

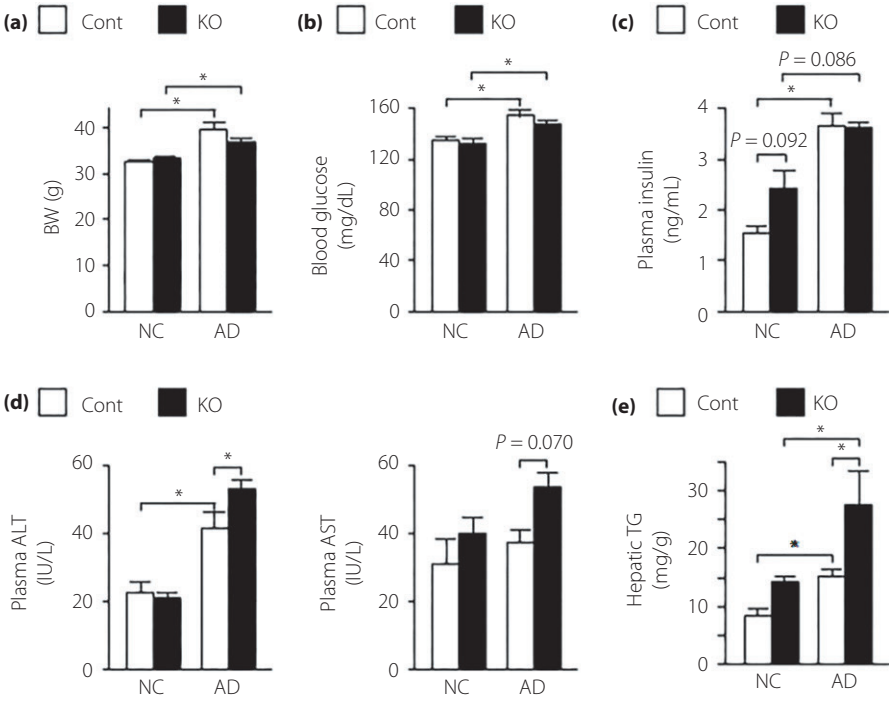

Figure 1 | (a) Body weight (BW), (b) blood glucose, (c) plasma insulin, (d) plasma aspartate aminotransferase/alanine aminotransferase (ALT/AST) and (e) hepatic triglyceride (TG) content in mice fed normal chow (NC) or an atherogenic high-fat diet (AD) for 32 weeks. Values are the mean ± standard error of the mean (NC-control [Cont], $n = 4$; NC-knockout [KO], $n = 5$; AD-Cont, $n = 6$; AD-KO, $n = 10$). *$P < 0.05$.

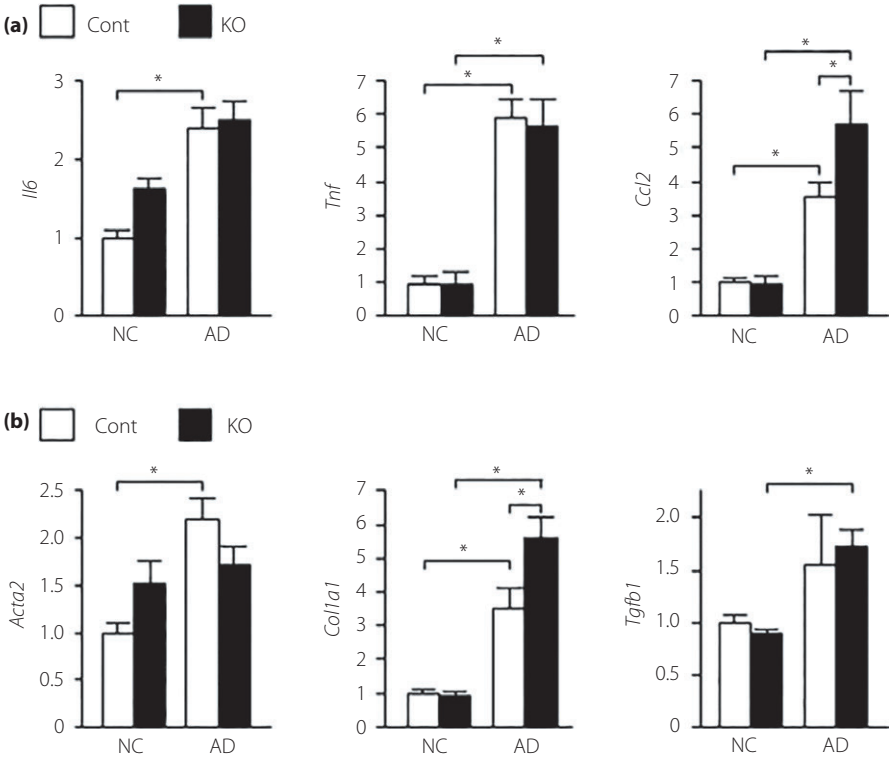

Figure 2 | Hepatic messenger ribonucleic acid expression levels of genes related to (a) inflammation and (b) fibrosis in mice fed normal chow (NC) or an atherogenic high-fat diet (AD) for 32 weeks. Values are the mean ± standard error of the mean (NC-control [Cont], $n = 4$; NC-knockout [KO], $n = 5$; AD-Cont, $n = 6$; AD-KO, $n = 10$). *$P < 0.05$.

α7KO mice fed an MCD compared with α7KO mice fed a control diet, whereas there was no change in the hepatic expression of M2 marker genes between the groups (Figure 5a, b and Figure S2c). α7KO mice showed higher expression of *Tnf* and *Tgfb1* genes, and exacerbation of liver fibrosis on hepatic histology with Sirius red stain compared with control mice (Figure 5a–c).

DISCUSSION

Along with hepatic fat accumulation as a result of insulin resistance, hepatic inflammation is deeply involved in the onset and progression of NASH[1,7]. α7nAchR in the vagus nerve plays an important role in the regulation of hepatic inflammation, but this vagal regulation is impaired in individuals with insulin resistance[12]. Although previous studies have reported that acute or chronic α7nAchR impairment exacerbates hepatic inflammation in NAFLD[13,24,25], no previous study has elucidated the effect of α7nAchR impairment on the progression of NASH; that is, the exacerbation of not only inflammation, but also fibrosis. In the present study, using α7KO mice and diet-induced animal models of NASH, we found that α7nAchR impairment leads to both inflammation and fibrosis related to the exacerbation of NASH. In AD- and MCD-fed mice, α7nAchR deficiency induced elevation of plasma transaminase levels, markers of liver injury; upregulation of genes associated with inflammation and fibrosis; and an increase in fibrosing lesions on liver histology.

In the present study, we used AD and MCD to establish two diet-induced NASH models with distinct mechanisms, and showed that α7nAchR deficiency exacerbates both NASH-related inflammation and fibrosis. In particular, we found that α7nAchR deficiency resulted in hepatic pericellular fibrosis in collagen fiber staining, in addition to the increase in the expression of inflammation- and fibrosis-associated genes. Previous studies have reported the association of α7nAchR deficiency with the exacerbation of hepatic inflammation caused by bacterial endotoxins[13] and of inflammation induced by HFD or short-term MCD loading[24,25]. However, HFD or short-term MCD loading is insufficient to induce liver fibrosis, making it difficult to clarify the role of α7nAchR impairment in liver fibrosis, although liver fibrosis, together with hepatic inflammation, is a major manifestation of NASH[26]. The present study, by examining liver fibrosis in tissue sections stained with Sirius red after the administration of AD and MCD for 32 and 6 weeks, respectively, has shown that α7nAchR deficiency exacerbates liver fibrosis in NASH. AD and MCD are widely used to establish a diet-induced animal model of NASH[2,17]. The AD model is thought to closely represent the pathology of NASH in humans, because the diet induces and promotes insulin resistance and hepatic fat accumulation, eventually triggering

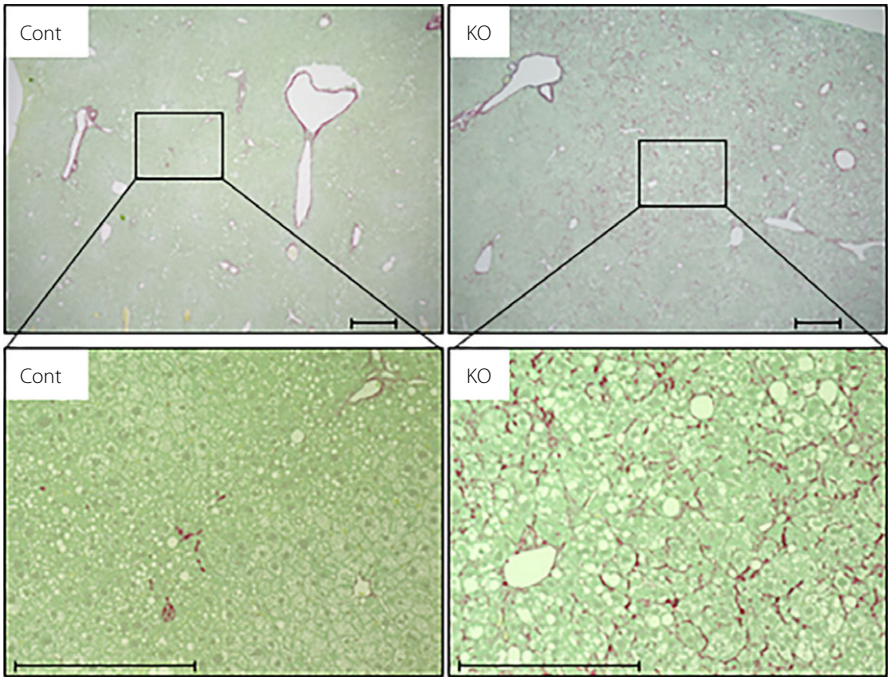

Figure 3 | Hepatic fibrosis in mice fed an atherogenic high-fat diet for 32 weeks, evaluated using representative Sirius red-stained histological sections of the liver. Scale bar, 500 μm. Cont, control; KO, knockout.

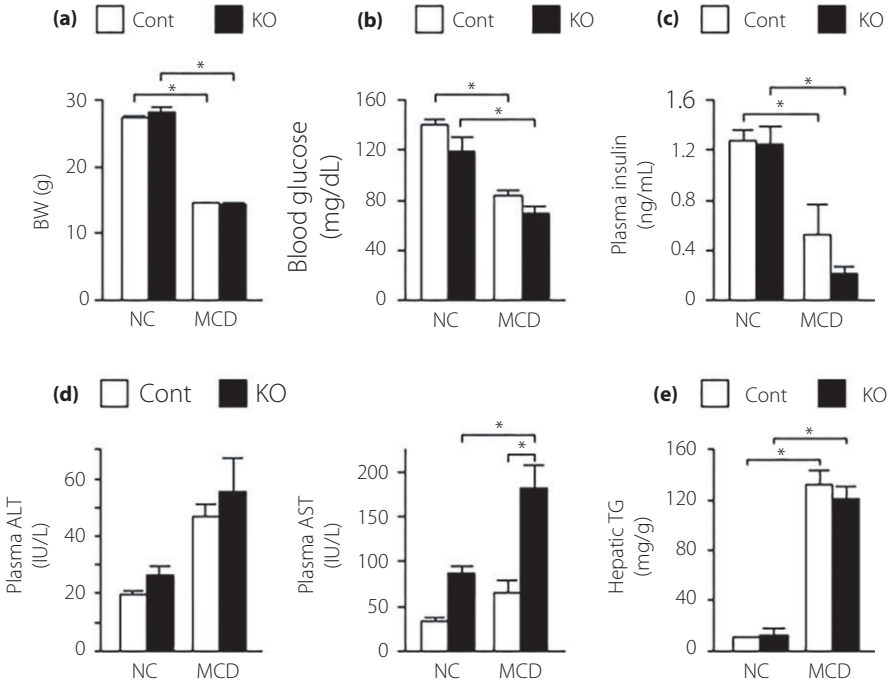

Figure 4 | (a) Body weight (BW), (b) blood glucose, (c) plasma insulin, (d) plasma aspartate aminotransferase/alanine aminotransferase (ALT/AST) and (e) hepatic triglyceride (TG) content in mice fed normal chow (NC) or a methionine/choline-deficient diet (MCD) for 6 weeks. Values are the mean ± standard error of the mean (NC-control [Cont], $n = 4$; NC-knockout [KO], $n = 4$; MCD-Cont, $n = 6$; MCD-KO, $n = 8$). *$P < 0.05$.

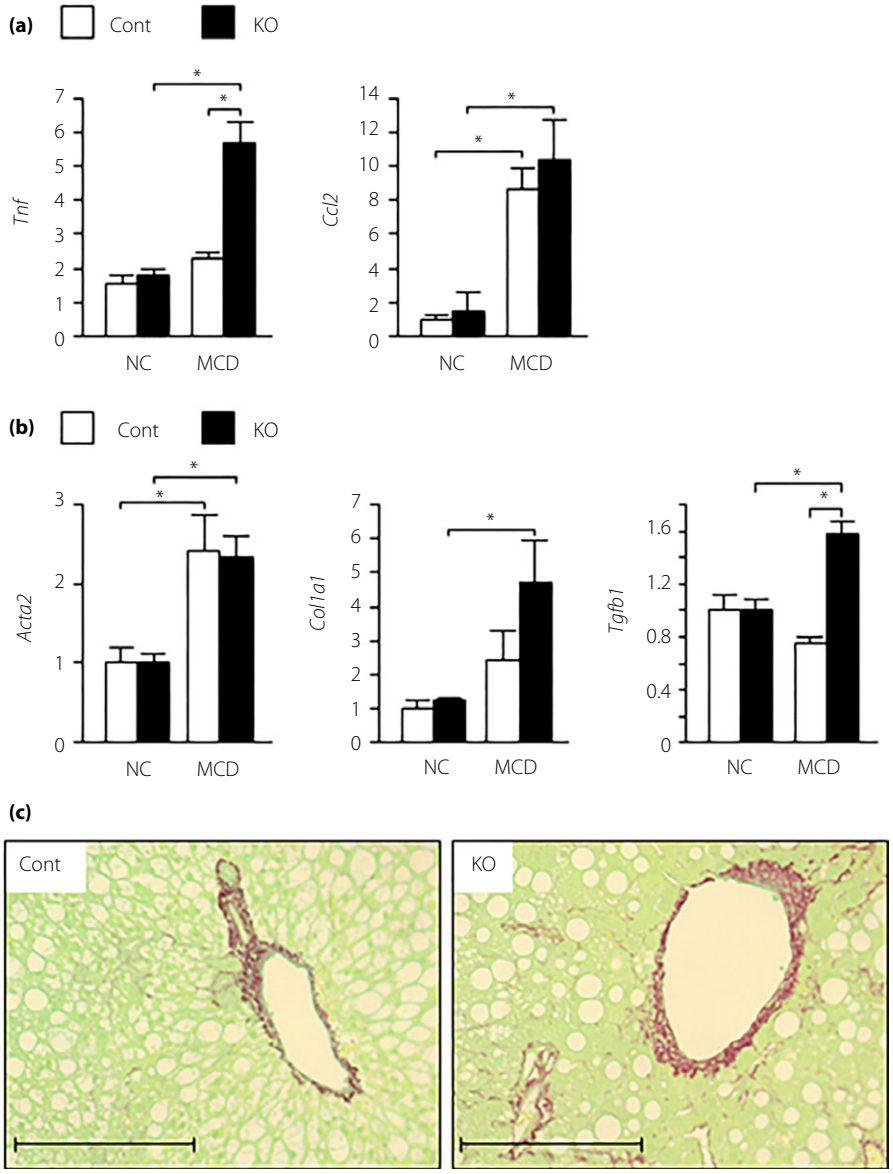

Figure 5 | Hepatic expression of genes related to (a) inflammation and (b) fibrosis, and (c) liver histological sections of Sirius red staining in mice fed normal chow (NC) or a methionine/choline-deficient diet (MCD) for 6 weeks. Values are the mean ± standard error of the mean (NC-control [Cont], $n = 4$; NC-knockout [KO], $n = 4$; MCD-Cont, $n = 6$; MCD-KO, $n = 8$). *$P < 0.05$. Scale bar, 500 μm.

inflammation and fibrosis in the liver[2,17]. In contrast, the MCD model triggers NASH through hepatic fat accumulation caused largely by impaired release of hepatic triglycerides as a result of the lower synthesis of VLDL[2,17]. Indeed, animals fed an MCD have substantial weight loss. and reduced blood glucose and plasma insulin levels. The MCD model showed no difference in the hepatic triglyceride concentration between α7KO mice and their control, whereas AD loading exacerbated hepatic steatosis in α7KO mice. This phenotypic difference between MCD and AD might depend on the difference in the effect on plasma insulin levels. Scherer *et al.* reported that central insulin action regulates VLDL secretion and reduces hepatic triglyceride content, although the mechanism underlying the central insulin regulation of VLDL secretion remains unclear.[27] α7nAchR plays an important role in brain–liver interaction through the vagus nerve[12]. If α7nAchR also affects central insulin-mediated regulation of VLDL secretion, α7nAchR deficiency could increase the hepatic triglyceride concentration.

After 18-week administration of HFD, α7KO mice have impaired glucose tolerance accompanied by insulin resistance and high fasting blood glucose levels[14]. The HFD model induces severe insulin resistance and hepatic inflammation, although the animals lack clear signs of liver fibrosis[2,17]. In the present study, even though blood glucose and plasma insulin

levels increased after AD administration, no significant difference was observed between control and α7KO mice, suggesting that the mechanism underlying the exacerbation of inflammation/fibrosis as a result of α7nAchR deficiency is different from the mechanism underlying the impairment of glucose tolerance and the exacerbation of insulin resistance. Insulin resistance induced by the AD is reportedly milder than that induced by the HFD[2], and this might explain why AD-fed α7KO mice did not have clear signs of hyperglycemia or hyperinsulinemia in the present study.

According to the Mouse Expression Database, α7nAchR is expressed in the nervous system and hemolymphoid system[28]. Our previous study also showed the expression of α7nAchR in the central nervous system and Kupffer cells[12]. In α7KO mice, exacerbation of diet-induced hepatic inflammation is likely mediated by α7nAchR in inflammatory cells in the liver, such as Kupffer cells. We have reported that α7KO mice show an increase in hepatic inflammatory responses, and that bone marrow-specific restoration of α7nAChR in α7KO mice results in amelioration of the increased hepatic inflammatory response[12]. Furthermore, hepatic inflammation induced by short-term administration of MCD is exacerbated in bone marrow-derived cell-specific α7nAChR knockout mice generated by transplantation of α7KO bone marrow to wild-type mice[24].

The exacerbation of diet-induced liver fibrosis in α7KO mice might be attributable to persistent severe hepatic inflammation, instead of being mediated by α7nAchR in stellate cells, the major player in liver fibrosis. This is because an immunohistological study has shown that α7nAchR is less abundantly expressed in hepatic stellate cells[29]. However, a future challenge would necessarily be to elucidate the functional role of α7nAchR in stellate cells and macrophage cells in hepatic fibrosis in NASH by using stellate cell- and macrophage-specific α7nAchR knockout mice. AD administration increased *Col1a1* gene expression in α7KO mice, but not *Tgfb1* and *Acta2* gene expression. Meanwhile, MCD administration increased *Tgfb1* gene expression, although *Col1a1* gene expression showed a tendency for an increase in α7KO mice. The difference in the fibrosis-associated gene expression pattern might be explained by the timing of the liver sample harvest, because both AD and MCD models showed hepatic fibrosis in collagen fiber staining.

The present study shows that the functional deficit of α7nAchR exacerbates both inflammation and fibrosis attributable to hepatic fat accumulation. Given that insulin resistance impedes vagal regulation of inflammation, insulin resistance might develop and exacerbate NASH through the impairment of vagal α7nAchR activity. α7nAchR agonists alleviate acute inflammatory diseases in the liver, such as toxic liver damage and ischemic liver injury[30,31], and improve chronic hepatic inflammation induced by HFD[25]. α7nAchR might be a novel drug candidate for the prevention and treatment of NASH, but further study is required to verify the utility of α7nAchR agonists for liver fibrosis in NASH. Because α7nAchR is abundant in the central nervous system, α7nAchR agonists might be promising candidate therapeutic targets for cognitive disorders, such as schizophrenia and Alzheimer's disease[32,33]. The application of activated α7nAchR in the prevention and treatment of NASH requires further investigation of α7nAchR action in organs other than the liver, and the development of a liver-specific drug delivery system for α7nAchR agonists.

ACKNOWLEDGMENTS

We thank K Nagamori and C Asahi (Kanazawa University) for providing technical assistance, and ThinkSCIENCE (Tokyo, Japan) for help with the preparation of the manuscript. This work was supported by the Japan Society for the Promotion of Science KAKENHI (grant number 15H05678 to Y Inaba, grant number 16K01815 to K Kimura, and grant number 17H05499 to H Inoue); a research grant from the Naito Foundation (to K Kimura); and a research grant from the Uehara Memorial Foundation and the Takeda Science Foundation (to H Inoue).

DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1| Hepatic messenger ribonucleic acid expression levels of genes related to (a) lipogenesis, (b) β-oxidation and very high-density lipoprotein (VLDL) secretion, and macrophage polarization markers in mice fed a normal chow (NC) or an atherogenic high-fat diet (AD)for 32 weeks.

Figure S2| Hepatic messenger ribonucleic acid expression levels of genes related to (a) lipogenesis, (b) β-oxidation and VLDL secretion, and macrophage polarization markers in mice fed a normal chow (NC) or a methionine/choline-deficient diet (MCD) for 6 weeks.

Table S1| Primer sequences.