Original Article

p59fyn is associated with the development of hepatic steatosis due to chronic ethanol consumption

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p59fyn, a protein tyrosine kinase belonging to the src-family, is involved in the regulatory mechanism of acute response to ethanol in the central nervous system. A previous report showed an association between src-family kinase activity and fatty acid oxidation, and it also reported that hepatic free fatty acid levels were low in Fyn–/– mice. We examined, using Fyn–/– mice whether Fyn is also involved in fatty acid metabolism and the development of pathological changes in the liver in response to chronic ethanol consumption. C57BL/6J Fyn–/– and Fyn+/+ mice were fed for 8 weeks with either a liquid diet comprising ethanol or one in which the calories from ethanol were replaced with carbohydrates. Chronic ethanol consumption for 8 weeks resulted in remarkable hepatic steatosis in Fyn+/+ mice but not in Fyn–/– mice. Chronic ethanol consumption induced a significant decrease in hepatic FFA and triglyceride levels in Fyn–/– mice. Levels of interleukin-6, which is associated with the enhancement of fatty acid oxidation, was also increased significantly in the livers of ethanol-fed Fyn–/– mice. The results suggest that Fyn is involved in the enhancement of fatty acid oxidation and the development of hepatic steatosis caused by chronic ethanol consumption.

***Key Words*: p59fyn, chronic ethanol consumption, fatty acid oxidation, interleukin-6, steatosis**

P59fyn (Fyn) belongs to the src-family tyrosine kinase and is ubiquitously expressed in various organs, most abundantly in the immune system.[1] Fyn is known to play an important role in T cell receptor-mediated apoptosis.[2–7]

Fyn has also been shown to be involved in the ethanol-mediated acute response in the central nervous system.[8] Fyn–/– mice were previously shown to be sensitive to hypnotic effects of ethanol, and tyrosine phosphorylation of the hippocampal N-methyl-d-aspartate (NMDA) type 2B receptor by Fyn plays an important role in these effects.[8] In addition, Fyn–/– mice showed enhanced fatty acid oxidation, and Fyn kinase activity was found to be involved in the regulation of fatty acid oxidation.[9]

Ethanol is the most widely abused substance in the world. Chronic alcoholism is known to cause pathological changes in several organs, such as the liver, primarily because of disorders in fatty acid oxidation in the liver.

Because Fyn has been reported to play an important role in acute response to ethanol in the brain and to regulate fatty acid oxidation in peripheral tissues under normal dietary conditions, we examined the association of Fyn with the development of hepatic steatosis induced by chronic ethanol consumption.

Materials and Methods

Animals. C57BL/6J Fyn–/– (Fyn–/–) mice were provided by Jackson Laboratories (Bar Harbor, ME). This strain lacks the normal expression of both brain- and lymphocyte-specific forms of Fyn.[10] C57BL/6J Fyn+/+ (Fyn+/+) mice were obtained from Japan SLC Co. Ltd. (Shizuoka, Japan). Age-matched 8-week-old male mice were used for the experiments. They were housed in plastic cages for 8 weeks on a 12-h light/12-h dark cycle under controlled temperature (22 ± 1°C) and humidity (50 ± 10%).

Experimental designs. Mice were fed a liquid diet containing ethanol (Lieber/DeCarli liquid diet-ethanol) or a liquid diet in which the calories from ethanol were replaced with carbohydrates (Lieber/DeCarli liquid diet-control), which was obtained from Dyet Co. Ltd. (Bethlehem, PA). The mice were divided into the following four experimental groups: (i) control-fed Fyn+/+ mice ($n = 6$), (ii) ethanol-fed Fyn+/+ mice ($n = 6$), (iii) control-fed Fyn–/– mice ($n = 6$), and (iv) ethanol-fed Fyn–/– mice ($n = 6$). All mice were fed for 8 weeks under this condition. Following this, the mice were starved overnight, and their livers and bloods were removed for further experiments. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Osaka Medical College, Prefecture, Japan). Each set of 8-week feeding experiments was carried out twice.

Measurement of serum biochemical parameters. Levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by a local laboratory for clinical examinations (SRL Co. Ltd., Tokyo, Japan).

Measurement of hepatic biochemical parameters. Hepatic tissues were homogenized using a Polytron homogenizer (ULTRA-TURRAX TP18/1051; Janke & Kunkel, IKA-Labortechnik, Staufeni, Germany). Homogenization was done in a buffer of pH 7.4 containing 20 mM Tris HCl, 1 mM EGTA, 2 mM EDTA, and protease inhibitor cocktail containing leupeptin (2 μg/mL). Hepatic tissue free fatty acid (FFA), triglyceride (TG), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) levels were measured by SRL Co. Ltd. (Tokyo, Japan).

Histological analysis. The livers were formalin-fixed, paraffin-embedded, and processed for hematoxylin and eosin (H&E) staining. Fatty changes in livers were classified into 4 grades: <30% (0), 30–50% (1+), 50–70% (2+), and >70% (3+) hepatic steatosis in lobules, as previously reported with some modifications.[11] Fatty deposits were verified by staining with Oil Red O

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Immunohistological analysis. For immunohistological analysis, the ABC staining kit (Vector Labs, Burlingame, CA) was used in combination with anti-p59fyn antibody (Santa Cruz, CA).

Statistical analysis. The significance of differences between values was analyzed using an unpaired Student's *t* test, Kruskal-Wallis test, and Mann-Whitney test. Differences were considered significant at $p<0.05$.

Results

8- and 16-week-old male Fyn+/+ and Fyn–/– mice were divided into four groups and fed a liquid diet-ethanol or a liquid diet-control for 8 weeks: (i) control-fed Fyn+/+ mice ($n = 6$), (ii) ethanol-fed Fyn+/+ mice ($n = 6$), (iii) control-fed Fyn–/– mice ($n = 6$), and (iv) ethanol-fed Fyn–/– mice ($n = 6$).

Effect of treatments with liquid diets on body weight and diet consumption. As shown Fig. 1, body weight of control-fed mice was not significantly different at the end of the experiment period (37.7 ± 4.4 and 34.8 ± 1.7 g in Fyn+/+ and Fyn–/– mice, respectively; $p<0.05$). Body weight of ethanol-fed mice was also not significantly different (28.0 ± 0.3 and 27.4 ± 1.4 g in Fyn+/+ and Fyn–/– mice, respectively; $p<0.05$). Significant differences were observed between the two groups with different diets but same genetic background (control-fed Fyn+/+ vs ethanol-fed Fyn+/+ mice; control-fed Fyn–/– vs ethanol-fed Fyn–/– mice, respectively; $p<0.05$).

As shown in Tables 1 and 2, daily diet consumption by same diet-fed mice was not significantly different ($p<0.05$).

Serum and hepatic biochemical parameters. To examine whether the two diets induced liver damage and steatosis, we quantified serum levels of AST and ALT and hepatic levels of FFAs and TGs. Serum AST and ALT levels were significantly higher in control-fed Fyn–/– mice than in control-fed Fyn+/+ mice ($p<0.05$) (Table 1). In contrast, serum AST and ALT levels were significantly higher in ethanol-fed Fyn+/+ mice than in ethanol-fed Fyn–/– mice ($p<0.05$) (Table 2). Serum AST and ALT levels were compared between the groups and were found to be significantly higher in ethanol-fed Fyn+/+ mice (about 1.2–1.3 and 1.2–1.9 times, respectively). Hepatic FFA and TG levels were significantly higher in control-fed Fyn+/+ mice than in control-fed Fyn–/– mice ($p<0.05$) (Table 1). In addition, hepatic FFA and TG levels in ethanol-fed Fyn+/+ mice were significantly higher than those in ethanol-fed Fyn–/– mice ($p<0.05$) (Table 2). Hepatic FFA and TG levels were compared between the groups and were

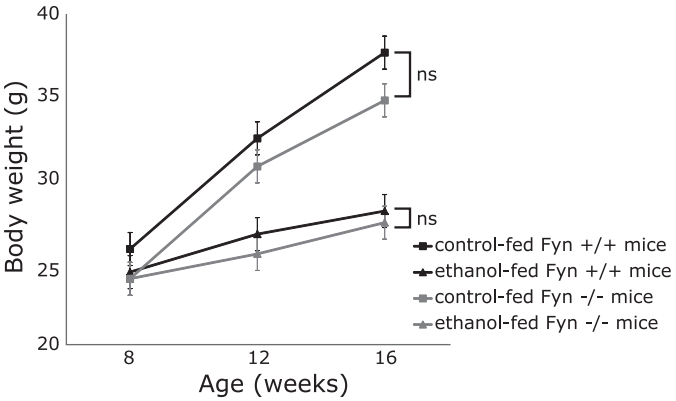

Fig. 1. Effects of treatments with liquid diets on body weight. Data are shown as the mean ± SD. ns: not significant.

Table 1. Diet consumptions and serum and hepatic biochemical parameters of the control-fed Fyn+/+ and Fyn–/– mice

	Fyn+/+ ($n = 6$)	Fyn–/– ($n = 6$)
Diet consumption (cc/day)	10.0 ± 0.9	10.2 ± 1.0
AST (IU/L)	271.8 ± 6.0	286.4 ± 9.3*
ALT (IU/L)	49.1 ± 2.0	63.1 ± 3.8*
Hepatic FFAs (μEQ/L)	40.3 ± 1.3*	34.9 ± 1.9
Hepatic TGs (mg/dL)	40.8 ± 4.5*	29.3 ± 2.8

AST, aspartate aminotransferase; ALT, alanine aminotransferse; FFAs, free fatty acids; TGs, triglycerides.
Statistical comparisons were made using Student's *t* test.
Differences were considered significant when *$p<0.05$.

Table 2. Diet consumptions and serum and hepatic biochemical parameters of the ethanol-fed Fyn+/+ and Fyn–/– mice

	Fyn+/+ ($n = 6$)	Fyn–/– ($n = 6$)
Diet consumption (cc/day)	6.0 ± 0.9	5.5 ± 0.9
AST (IU/L)	366.3 ± 8.9*	313.5 ± 7.6
ALT (IU/L)	91.9 ± 6.6*	77.9 ± 5.0
Hepatic FFA (μEQ/L)	56.7 ± 4.9*	24.8 ± 4.8
Hepatic TG (mg/dL)	156.3 ± 7.1*	15.3 ± 3.3

AST, aspartate aminotransferase; ALT, alanine aminotransferse; FFAs, free fatty acids; TGs, triglycerides.
Statistical comparisons were made using Student's *t* test.
Differences were considered significant when *$p<0.05$.

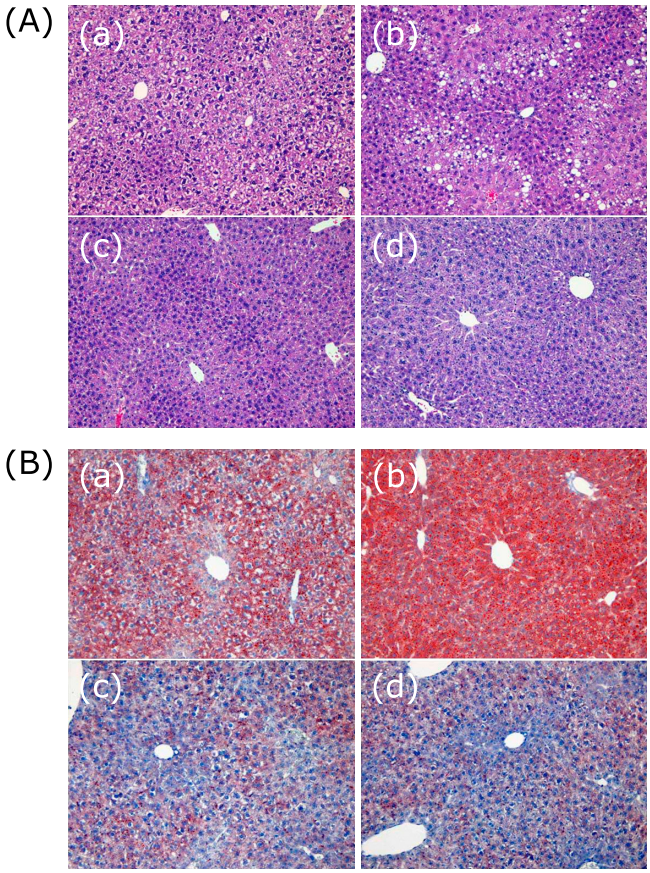

Fig. 2. Effects of a liquid diet containing ethanol or a liquid diet in which the calories from ethanol were replaced with carbohydrates (control) on the livers of Fyn+/+ and Fyn–/– mice. (A) Hematoxylin and eosin staining (original magnification ×150). (B) Oil Red O staining (original magnification ×150). Data are representative of two sets of experiments. (a) control-fed Fyn+/+ mice, (b) ethanol-fed Fyn+/+ mice, (c) control-fed Fyn–/– mice, and (d) ethanol-fed Fyn–/– mice.

found to be significantly higher in ethanol-fed Fyn+/+ mice (about 1.4–2.3 times and 3.8–10.2 times, respectively).

Histological analysis. Hepatic steatosis was distinctively observed in the livers of ethanol-fed Fyn+/+ mice (Fig. 2 (A)). In order to verify the existence of lipids, Oil Red O staining was performed. Fatty droplets were fewer in control-fed Fyn–/– mice when compared with control-fed Fyn+/+ mice (Fig. 2 (B) and Table 3). The existence of fatty droplets was significantly greater in ethanol-fed Fyn+/+ mice ($p = 0.0012$) than in the other groups. In contrast, such changes were lesser in the livers of ethanol-fed Fyn–/– mice (Fig. 2 (B) and Table 3).

Hepatic cytokine concentration. Levels of hepatic TNF-α and IL-6 were found to be significantly higher in control-fed Fyn–/– mice than in control-fed Fyn+/+ mice ($p<0.05$) (Fig. 3 (A)). Additionally, levels of hepatic TNF-α and IL-6 were found to be significantly higher in ethanol-fed Fyn–/– mice than in ethanol-fed Fyn+/+ mice ($p<0.05$) (Fig. 3 (B)). Consumption of the liquid diet containing ethanol resulted in significantly greater level of hepatic TNF-α in mice with the same genetic background (432.4 ± 24.1 and 655.0 ± 17.5 pg/mL in control-fed and ethanol-fed Fyn+/+ mice, respectively, and 563.2 ± 24.4 and 750.9 ± 21.7 pg/mL in control-fed and ethanol-fed Fyn–/– mice, respectively; $p<0.05$) (Fig. 3 (A) and (B)). In addition, the liquid diet with ethanol resulted in significantly greater levels of hepatic IL-6 in mice with the same genetic background (25.7 ± 0.8 and 34.0 ± 2.2 pg/mL in control-fed and ethanol-fed Fyn+/+ mice, respectively, and 46.7 ± 1.4 and 53.8 ± 2.0 pg/mL in control-fed and ethanol-fed Fyn–/– mice, respectively; $p<0.05$) (Fig. 3 (A) and (B)).

Immunohistochemical analysis. The immunolocalization of Fyn in the liver was examined by using the anti-Fyn antibody and was markedly detected in hepatocytes (Fig. 4).

Table 3. Hepatic steatosis in mice

		Degree of hepatic steatosis			
	n	0	1+	2+	3+
Control-fed Fyn+/+ mice	6	3	3	0	0
Ethanol-fed Fyn+/+ mice*	6	0	0	1	5
Control-fed Fyn–/– mice	6	4	2	0	0
Ethanol-fed Fyn–/– mice	6	5	1	0	0

There was a significant difference ($p = 0.0012$) in the degree of hepatic steatosis among 4 groups verified by the Kruskal-Wallis test.
*Significantly different (p is equal to 0.0024, 0.0023, and 00019) from control-fed Fyn+/+, control-fed Fyn–/–, and ethanol-fed Fyn–/– mice, respectively, by the Mann-Whitney test.

Discussion

Using C57BL/6J Fyn–/– and Fyn+/+ mice, we examined the effects of chronic ethanol consumption in the liver. Histological analysis of mice livers showed significant differences between ethanol-fed Fyn–/– and Fyn+/+ mice. Conversely, no apparent histological differences were observed in the livers of control-fed Fyn–/– and Fyn+/+ mice. Remarkable hepatic steatosis was observed in ethanol-fed Fyn+/+ mice, but not in ethanol-fed Fyn–/– mice. Because of these histological differences, we

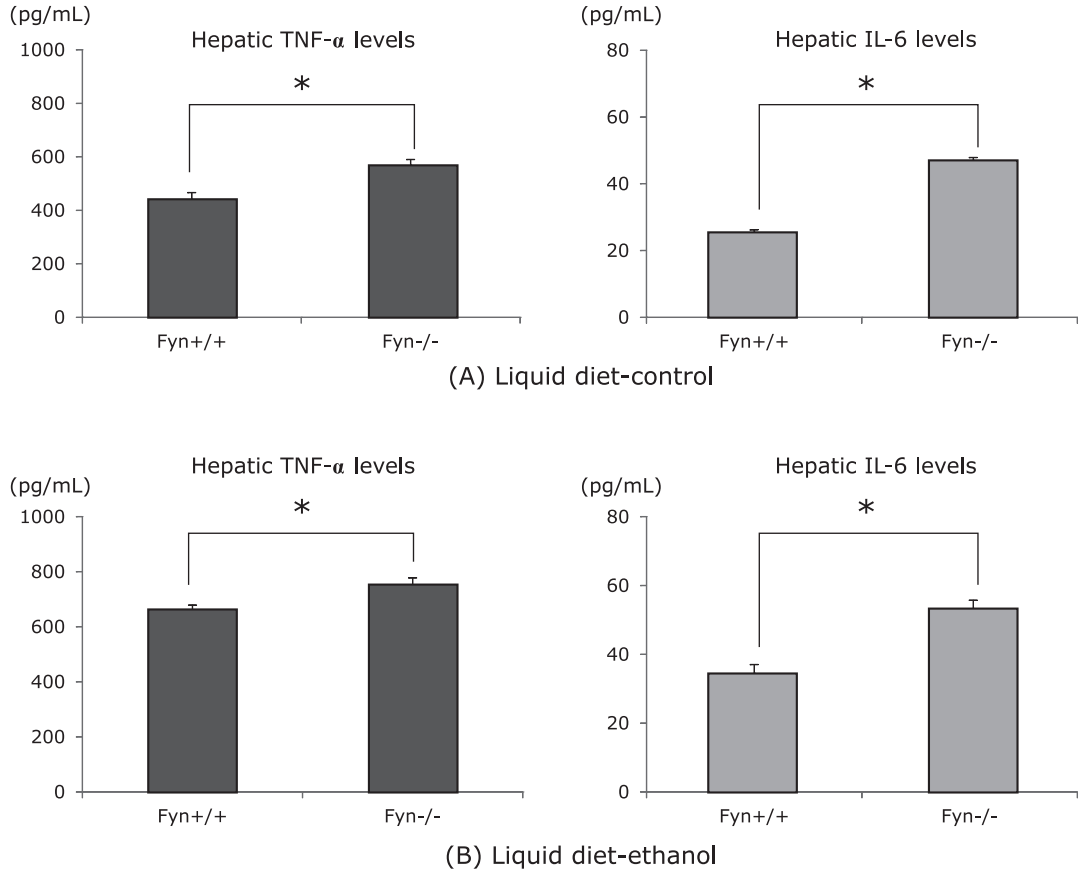

Fig. 3. Levels of TNF-α and IL-6 in the livers of Fyn+/+ ($n = 6$) or Fyn–/– ($n = 6$) mice. (A) Levels of hepatic TNF-α and IL-6 were measured in control-fed mice. Levels of hepatic TNF-α and IL-6 increased significantly in the livers of control-fed Fyn–/– mice. (B) Levels of hepatic TNF-α and IL-6 were measured in mice fed a liquid diet containing ethanol. Levels of hepatic TNF-α and IL-6 increased significantly in the livers of ethanol-fed Fyn–/– mice. Data are shown as the mean ± SD (*$p<0.05$).

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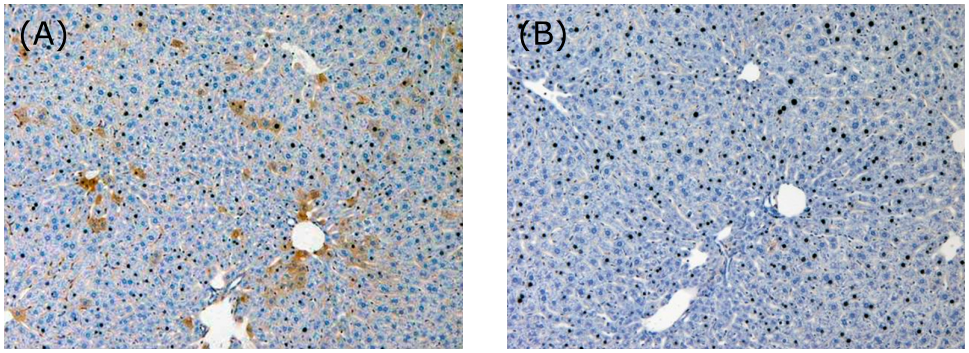

Fig. 4. Immunohistochemical localization of p59fyn in the liver. (A) anti-p59fyn antibody and (B) negative staining by omitting the primary antibody (original magnification ×150).

examined possible differences in serum markers of liver injury. Serum AST and ALT were significantly higher in ethanol-fed Fyn+/+ mice than in ethanol-fed Fyn–/– mice.

Interestingly, chronic ethanol consumption induced a significant decrease in the levels of hepatic FFAs and TGs in Fyn–/– mice than in the Fyn+/+ mice. These results coincided with the findings of the histological analysis of the livers of ethanol-fed Fyn–/– mice. Bastie *et al.* showed that the levels of hepatic FFAs and TGs were significantly decreased in Fyn–/– mice than in Fyn+/+ mice under normal dietary consumption.[9] The results of the present study concerning levels of hepatic FFAs and TGs in control-fed Fyn–/– mice also coincided with their results. In this study, the levels of hepatic FFAs and TGs were significantly lower in ethanol-fed Fyn–/– mice than in control-fed Fyn–/– mice. These results suggested that a Fyn deficiency was also involved in an enhancement of fatty acid oxidation during ethanol metabolism.

It is well known that serum levels of the proinflammatory cytokines TNF-α and IL-6 are elevated in alcoholics and in animals fed ethanol chronically.[12–15] Another interesting finding of this study was that the production of these proinflammatory cytokines was significantly greater in the livers of ethanol-fed Fyn–/– mice than in the livers of ethanol-fed Fyn+/+ mice. It was of interest that the production of these cytokines in the liver was also significantly different between control-fed Fyn–/– and Fyn+/+ mice. These results suggest that Fyn is associated with the regulation of hepatic TNF-α and IL-6 production.

Excess TNF-α production has shown to play an important role in most forms of experimental liver disease.[15–17] In the present study, the increase in the production of hepatic TNF-α in ethanol-fed Fyn+/+ and Fyn–/– mice also coincided with the elevation of serum AST and ALT markers of liver injury.

IL-6 has also been shown to play an important role in liver regeneration, repair from injury, and antiapoptosis.[18–20] Hong *et al.* reported that injection of IL-6 for 10 days prevented fatty liver in ethanol-treated mice.[11] Furthermore, they reported that the beneficial effect of IL-6 treatment was an increase in β-oxidation of mitochondrial fatty acids.[11] In this study, ethanol-fed Fyn–/– mice showed an increase in the production of hepatic IL-6 and did not show the development of hepatic steatosis. Therefore, increase in the production of IL-6 in the livers of Fyn–/– mice might be due to enhanced fatty acid oxidation, resulting in inhibition of histological changes due to chronic ethanol consumption. Despite increased production of hepatic TNF-α in ethanol-fed Fyn–/– mice when compared to that in ethanol-fed Fyn+/+ mice, the elevation in serum AST and ALT levels in Fyn–/– mice was inhibited when compared with levels in Fyn+/+ mice. Inhibition of elevation in these serum parameters might have also been associated with the increase in production of IL-6 in the liver of Fyn–/– mice.

Because immunohistochemical analysis revealed the expression of Fyn in hepatocytes, Fyn signaling in the cells may be strongly associated with the mechanism responsible for greater ethanol tolerance in Fyn–/– mice. Kupffer cells and hepatocytes produce IL-6[21,22]; therefore, the results of this study suggest that a Fyn deficiency may, at least in part, increase IL-6 level in the liver.

Alcohol is the most common etiological factor for fat accumulation in hepatocytes, which is also the earliest and most common response to alcohol.[23,24] Therefore, inhibition of this response is clinically important. We showed herein that chronic ethanol consumption induced the enhancement of fatty acid oxidation as a result of an increase in IL-6 production in the livers of Fyn–/– mice, which resulted in the resistance to hepatic steatosis in these mice. In conclusion, Fyn signaling might regulate the development of hepatic steatosis due to chronic ethanol consumption.

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Abbreviations

Fyn	p59fyn
FFA	Free Fatty Acid
TG	Triglyceride
Fyn–/–	C57BL/6J Fyn–/–
Fyn+/+	C57BL/6J Fyn+/+
AST	Aspartate Aminotransferase
ALT	Alanine Aminotransferase
TNF-α	Tumor Necrosis Factor-α
IL-6	Interleukin-6
H&E	Hematoxylin And Eosin

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