Original Article

Nonalcoholic Fatty Liver Disease Progression in Rats is Accelerated by Splenic Regulation of Liver PTEN/AKT

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ABSTRACT

Background/Aim: The spleen has been reported to participate in the development of nonalcoholic fatty liver disease (NAFLD), but the mechanism has not been fully characterized. This study aims to elucidate how the spleen affects the development of NAFLD in a rat model. **Materials and Methods:** Following either splenectomy or sham operation, male Sprague–Dawley (SD) rats were fed a high-fat diet to drive the development of NAFLD; animals fed a normal diet were used as controls. Two months after surgery, livers and blood samples were collected. Serum lipids were measured; liver histology, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) gene expression, and the ratio of pAkt/Akt were determined. **Results:** Splenectomy increased serum lipids, except triglyceride (TG) and high-density lipoprotein (HDL), in animals fed either a high-fat or normal diet. Furthermore, splenectomy significantly accelerated hepatic steatosis. Western blot analysis and real-time polymerase chain reaction showed splenectomy induced significant downregulation of PTEN expression and a high ratio of pAkt/Akt in the livers. **Conclusions:** The spleen appears to play a role in the development of NAFLD, via a mechanism involving downregulation of hepatic PTEN expression.

Key Words: Hyperlipidemia, nonalcoholic fatty liver disease, PTEN, spleen, splenectomy

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Nonalcoholic fatty liver disease (NAFLD), which is commonly associated with obesity, diabetes, and metabolic syndrome, is becoming increasingly prevalent worldwide. Histological features of NAFLD range from hepatic steatosis to nonalcoholic steatohepatitis (NASH), often resulting in liver fibrosis and cirrhosis, which can lead to the development of hepatocellular carcinoma.[1-3] The "two-hit theory" of NAFLD development, proposed by Day *et al.*, 1998,[4] is presently the most widely accepted model. In this model, the first "hit"—lipid peroxidation in the liver—leads to hepatic steatosis. A second "hit," such as the accumulation of reactive oxygen species, results in the development of NASH.[5] The precise molecular mechanisms controlling NAFLD-associated pathogenesis; however, have not been fully elucidated.

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Several studies have explored the relationship between lipid metabolism, which is central to the development of the NAFLD, and the spleen. Both *in vitro* and *in vivo* studies have demonstrated that the spleen plays an important role in lipid metabolism, with splenectomy inducing hyperlipidemia.[6-8] Recently, Oishi *et al.*, 2011, and Inoue *et al.*, 2012, found that splenectomy exacerbates triglyceride (TG) deposition in the liver, suggesting a role for the spleen in preventing progression of hepatic steatosis to steatohepatitis.[9-11] The specific mechanism, by which the spleen exerts control over fatty acid metabolism in the liver, however, has not been described.

In the process of establishing a mouse model of NASH, several groups discovered a relationship between phosphatase and tensin homologue deleted on chromosome 10 (PTEN) activity in the liver and steatosis. In particular, liver-specific PTEN knockout mice developed steatosis similar to that observed in NASH, suggesting a potential role for this molecule in progression to NAFLD.[12,13] PTEN is a tumor suppressor and well-known negative regulator of the Akt signaling pathway, activation of which is a hallmark of steatohepatitis.[14-17] Correspondingly, hepatic PTEN expression is downregulated in both animal

models of NAFLD and patients with liver steatosis, and liver-specific inhibition of PTEN *in vitro* facilitates accumulation of TGs, partially due to constitutive Akt signaling.[18]

To better characterize the molecular mechanism of splenectomy-associated NAFLD, we examined its effect on liver PTEN expression, *in vivo*. PTEN expression was assessed two months after splenectomy in a rat model of NAFLD, induced by a high-fat diet, to determine the role of the spleen in regulation of hepatic steatosis, mediated by Akt signaling.

MATERIALS AND METHODS

Animals and surgical procedures

Male Sprague–Dawley rats, weighing 160–180 g, were supplied by the animal center of Southern Medical University, Guang Zhou, China. All rats were housed in appropriate cages, at an ambient temperature of 25°C, on a 12-h light/dark cycle. The rats were allowed free access to chow and water. All animals were treated in accordance with the Southern Medical University Guidelines for the Care and Use of Research Animals.

Forty rats were fed adaptively for a week, after which they were randomly divided into four groups: Group 1—normal diet with sham operation; Group 2—normal diet with splenectomy; Group 3—high-fat diet with sham operation; Group 4—high-fat diet with splenectomy. The high-fat diet contained 20% lard, 1% cholesterol, and 79% fundamental rat-diet power, produced by the animal center of the Southern Medical University. Savard *et al.*, 2013, have shown that dietary fat and dietary cholesterol strongly interact in the development of both the hepatic histological abnormalities and other parameters, such as body weight, liver weight, liver weight/body weight ratio, hepatic lipid concentration, and plasma alanine transferase and blood lipid levels.[19] The effects of dietary fat and cholesterol together on these parameters were more than two times greater than the sum of the effects observed with either high dietary fat or high dietary cholesterol alone. However, neither factor alone is sufficient to cause NASH.[20]

Following an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and induction of deep anesthesia, splenectomy was aseptically performed through a left-sided, lateral, 2.0 cm subcostal incision. After ligation of splenic vessels with 4-0 silk sutures, the spleen was removed. The incision was then closed in two layers, using 4-0 nylon sutures. The sham operation was performed using the same incision, but the spleen was returned to the abdominal cavity without ligation; the incision was then closed in two layers, as above. After surgery, the rats were fed with either a normal diet or a high-fat diet, according to group designation, for two months. We monitored the animal's wellbeing by observing the mental health, mobility, and feeding status of animals after surgery. Because aseptic surgery was appropriately performed, all animals survived well during the experiments.

Measurements and blood sampling

After two months of either normal or high-fat diet, the rats were anesthetized with sodium pentobarbital, as above, and the weights were recorded. The postcaval vein was punctured to collect venous blood (5 mL), which was centrifuged at 2000 *g* for 10 min at 4°C, after standing at room temperature for two hours. The supernatant was stored at −80°C, for future lipid analysis. The whole liver was immediately removed and weighed. The mean time from removing the liver to placing it in −80°C was 5 min. One lobe, the same for each animal, was removed for pathology examination; the remaining lobe was stored at −80°C, for future protein and RNA extraction. Serum lipids, including TG, low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), and high-density lipoprotein (HDL), and total cholesterol (TC) were measured by an automated analyzer (Dade Behring RXL, Deerfield, IL, USA).

Histological examination

Liver tissues were fixed in 10% formalin, and embedded in paraffin. Sections (4 μm thick) were stained with hemotoxylin and eosin (H and E), and examined with Olympus DP71 microscope (Olympus, Tokyo, Japan).

Determination of total liver TGs

After the two-month experimental duration, liver TGs were extracted and detected, according to the method described by Folch *et al.*, 1957.[21] Briefly, liver tissues were homogenized in a 2:1 chloroform: Methanol solution. The resultant extract was then washed in a salt solution (added 20% volume), and the mixture was separated into two phases, according to hydrophobicity. The lower phase, which contained the lipids, was carefully removed, and the concentration of TGs was determined using a colorimetric diagnostic kit (Applygen, Beijng, China), according to the manufacturer's protocol.

Real-time reverse transcription polymerase chain reaction analysis

Total RNA was extracted from each liver using TRIzol (Invitrogen, Carlsbad, CA, USA), as per the manufacturer's instructions. Purified RNA plus random hexamers were used in the Transcriptor First Strand cDNA synthesis kit (Roche, Indianapolis, IN, USA) in order to synthesize cDNA. All cDNA was stored at −80°C prior to polymerase chain reaction (PCR). PCR was performed using the Quantitect SYBR Green PCR kit in a LightCycler (Roche). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. Primer sequences were as follows: For PTEN:

Forward: 5' AACTTGCAATTCCCCAGTTTG-TG3', reverse: 5'CCTTGTCA TTATCCGCACGC3'; for GAPDH: forward: 5'ACCACAGTCCATGCCATCAC3 '; reverse: 5'TCCACCACCACCCTGTTGCTGTA3'.

Western blot analysis

Frozen liver tissues (20–30mg) were homogenized in 200 μL of cell lysis buffer, vortexed, and incubated on ice for 30 min. Whole cell lysates were then centrifuged (15,000 *g*/min, 15 min, 4°C), and supernatants were collected. Concentration of the whole protein extract was determined using a commercially available BCA kit (Beyotime, Shanghai, China). Briefly, 40 μg of the protein extract was loaded onto a 12% SDS–polyacrylamide gel. The resultant electrophoresed bands were then transferred onto polyvinylidene fluoride (PVDF) membranes, followed by blocking in 5% BSA for 1 h at room temperature. The membranes were then washed in TBST [1 mL Tween-20 in 2 L Tris-buffered saline (TBS) buffer] three times. For identification of specific proteins, membranes were incubated with primary antibodies (5 μg/mL) to pAkt, Akt, PTEN, or β-actin (Abcam, London, Britain) at 4°C overnight. After being washed in TBST, as above, the membranes were incubated for 1 h at room temperature with fluorescent-conjugated secondary antibodies, and washed again as above, in the dark. The immunoreactive bands were quantified using a National Institutes of Health imaging software (Scion Corporation, Frederick, MD, USA).

Statistical analysis

All data were described as means ± standard deviation. Statistical analysis of the variation between the groups was determined by Student's *t*-test, using SPSS 19.0 (IBM, Armonk, New York, USA), where values of $P < 0.05$ were considered significant.

RESULTS

Effect of splenectomy and high-fat diet on liver/body weight ratios

Increases in liver mass are thought to be associated with the development of NASH. To determine the effect of splenectomy and/or diet on the ratio of liver-to-body weight, all animals and their livers were weighed at the conclusion of the two-month study period. As shown in Table 1, liver weight (LW, 11.68 ± 1.07 g vs. 14.04 ± 2.29 g, $P < 0.05$) and body weight (BW, 373.38 ± 13.96 g vs. 404.06 ± 16.04 g, $P < 0.05$) both significantly increased in the splenectomized rats on the high-fat diet, whereas only BW (378.71 ± 8.48 g vs 396.56 ± 15.19 g, $P < 0.05$) significantly increased in sham rats on the high-fat diet as compared with those on the normal diet. Overall, animals on identical diets failed to demonstrate a statistically significant difference in weights, regardless of which operative procedure they underwent [Table 1].

Effect of splenectomy on the rate of steatosis, induced by high-fat diet

To determine the effect of splenectomy on the development of steatosis from a high-fat diet, liver tissue histology and TG content were evaluated in rats fed a high-fat or normal diet for two months after receiving either splenectomy or sham operation. As shown in Figure 1, animals receiving a high-fat diet [Figure 1b and 1c] demonstrated significant steatosis, compared with livers obtained from animals fed a normal diet [Figure 1a]. Importantly, splenectomy resulted in greater severity of liver steatosis, compared with animals receiving sham surgery [Figure 1b and 1c]. As was observed for animals fed a normal diet after receiving a sham procedure [Figure 1a], animals receiving a normal diet following splenectomy did not demonstrate any sign of steatosis (data not shown).

Changes in lipid profiles following splenectomy

To further investigate the effect of splenectomy on steatosis induced by high-fat diet, we examined serum levels of TG, TC, LDL, VLDL, and HDL. As expected, rats receiving a high-fat diet had much higher concentrations of serum lipids (except for HDL), regardless of which surgery they underwent ($P < 0.05$, Figure 2). Splenectomy resulted in a significant increase in the concentrations of TC, LDL, and VLDL (only normal diet), but not TG and HDL, compared with the sham operation, both in the normal and high-fat diet groups (all $P < 0.05$, Figure 2).

To determine if similar effects were observable in the livers of these rats, we assessed total hepatic TGs. As shown in Figure 3, animals fed a high-fat diet demonstrated a significantly higher concentration of liver TGs, when compared with those fed a normal diet ($P < 0.05$). Splenectomy also resulted in significantly increased accumulation of liver TG in animals fed a high-fat diet, compared with those that underwent a sham operation ($P < 0.05$). Regardless of surgical procedure, no difference was noted for liver-TG concentrations in mice fed a normal diet [Figure 4].

Effect of splenectomy on hepatic PTEN expression

Several studies have suggested a role for PTEN in the development of NASH.[12,13] To assess whether PTEN mediates the observed steatosis following splenectomy in animals fed a high-fat diet, hepatic PTEN expression was

Table 1: Body weight, liver weight, and liver/body weight ratios

	ND+sham	ND+SPX	HF+sham	HF+SPX
BW (g)	378.71±8.48	373.38±13.96	396.56±15.19#	404.06±16.04#
LW (g)	12.76±1.39	11.68±1.07	13.91±2.10	14.04±2.29#
Ratio (%)	3.37±0.36	3.12±0.18	3.51±0.51	3.47±0.51

#*P*<0.05 compared to normal diet rats having undergone the same operation.
ND: Normal diet, HF: High-fat diet, Sham: Sham operation, SPX: Splenectomy

determined by western blot and real-time PCR (RT-PCR) analyses. In animals fed a high-fat diet, splenectomy resulted in a significant suppression of PTEN expression, compared with the sham operation group, both at the protein and mRNA levels ($P < 0.05$, Figure 4a).

As PTEN is a negative regulator of the Akt signaling pathway, we next sought to assess whether this inhibition of PTEN expression induced by splenectomy translated to constitutive activation of Akt. As shown in Figure 4b, we observed a significant increase in basal Akt activity ($P < 0.05$), in splenectomized rats, concomitant with decreased PTEN expression. However splenectomy failed to induce changes in Akt activity in animals fed a normal diet, (data not shown).

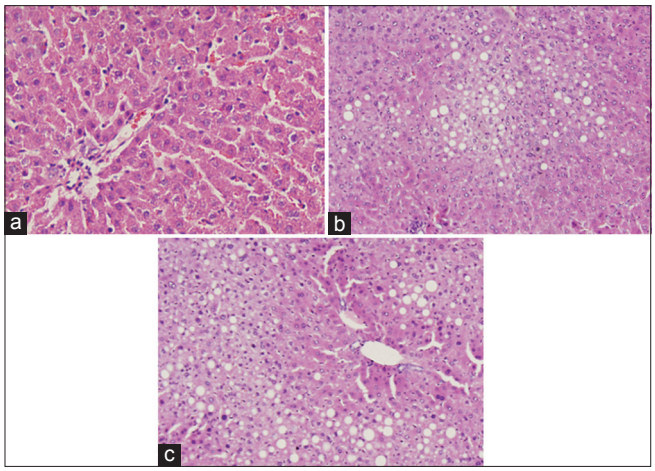

Figure 1: Splenectomy exacerbates steatosis in rats fed a high-fat diet. Hematoxylin and eosin staining (original magnification ×40) of livers from animals fed a normal diet + sham operation (a); a high-fat diet + sham operation (b); or a high-fat diet + splenectomy (c)

DISCUSSION

NAFLD is a growing health concern worldwide. Recent evidence suggests that the spleen may exert control over metabolic function, raising the possibility of its participation in the development of NAFLD.[20,22] Patients with myeloproliferative diseases associated with hypersplenism, such as polycythemia vera and myelofibrosis, demonstrate reduced TC, HDL and some apolipoproteins.[23,24] A study of World War II veterans, who underwent splenectomy, demonstrated a high incidence of acute myocardial infarction (MI).[8] It was proposed that the observed increase in MI might result from dyslipidemia, secondary to splenectomy. Several studies have been conducted to characterize splenic function in lipid metabolism.[6-8,20,25,26] Although results have been disparate, overall these studies imply a role for the spleen in regulation of lipid metabolism. Discrepancies between studies are likely attributable to differences in experimental design, including model species, study duration and diet, and even gender distribution, as Petroianu *et al*., used only female rats.[26] Interestingly, compared with the total splenectomy, partial splenectomy and hemisplectomy have been shown to result

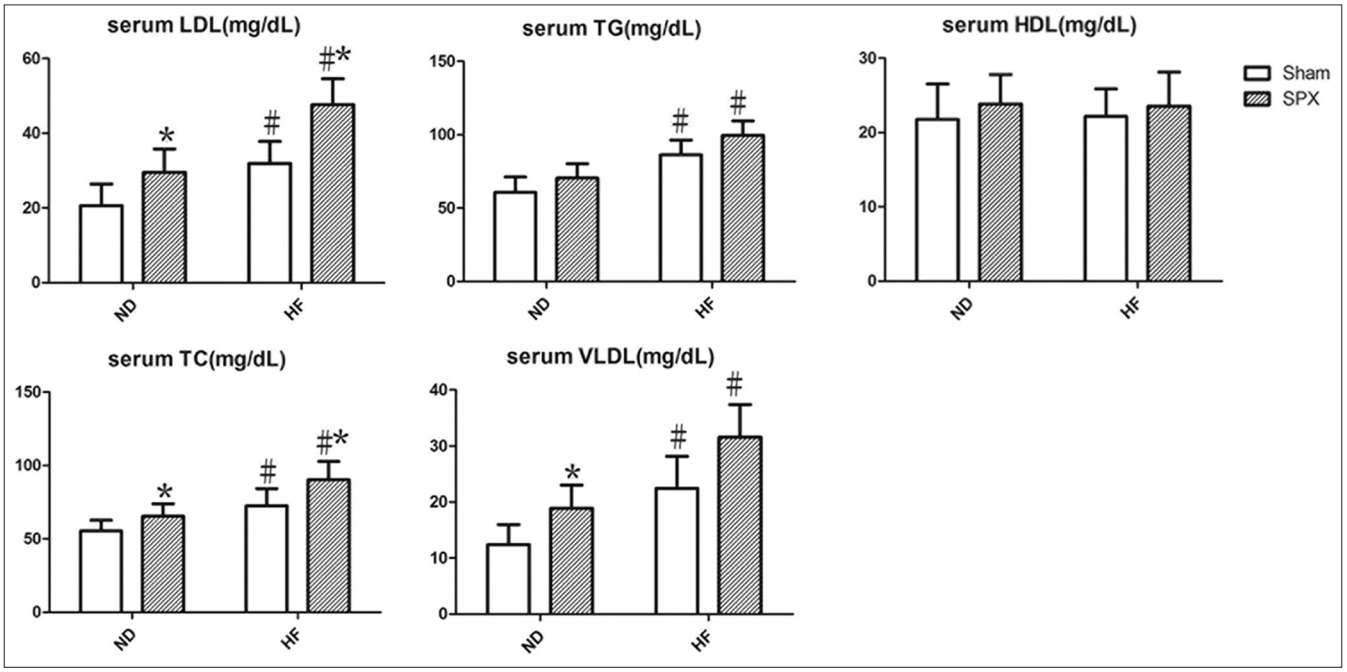

Figure 2: Splenectomy increases the concentration of serum lipids. Total lipids were extracted from the sera of animals and concentrations were determined. ND, normal diet; HF, high-fat diet; Sham, sham operation; SPX, splenectomy. $^{\#}P < 0.05$ compared with normal diet rats having undergone the same operation; $^{*}P < 0.05$ compared with rats having undergone sham operation

in more diminished lipid dysregulation, suggesting a direct correlation between spleen volume and function.[8,9,27]

In this study, sham rats on the high fat diet had a significantly higher serum TG, LDL, TC, VLDL, and liver TG level than those of rats on the normal diet, suggesting that high-fat diet worsened the lipid profile. Our data was consistent with previous studies.[8,9,26] And, we demonstrated that splenectomy worsened metabolism of TC, LDL, and VLDL, in animals fed either a normal or high-fat diet. Some theories have been raised to explain the possible mechanism of splenic regulation of lipid metabolism.[20,22,28] One theory has suggested that the spleen acts as a lipid reservoir, which would be exacerbated in the case of hypersplenism.[22] Due to an increase in phagocytic ability, spleen macrophages might accumulate large quantities of fat, resulting in hypolipidemia.[22] Indeed, some have suggested that diseases, including atherosclerosis, might result from autoimmune reactions against lipids.[29]

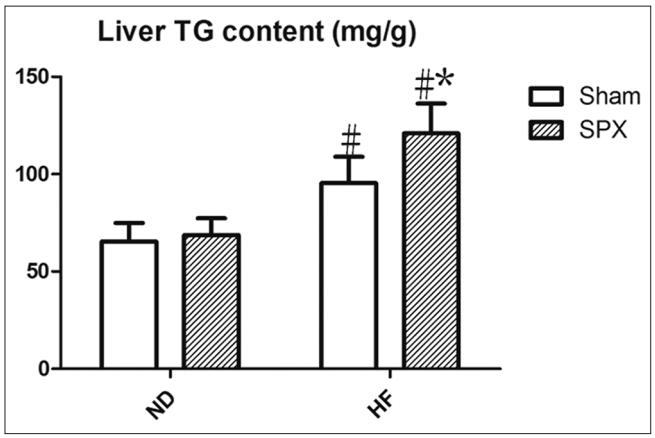

Figure 3: Splenectomy increases the concentration of liver triglyceride (TG) in animals receiving a high-fat diet. Total liver TGs were extracted from the sera of animals and concentrations were determined. $^{\#}P < 0.05$ compared with normal diet rats having undergone the same operation; $^{*}P < 0.05$ compared with rats having undergone sham operation

In addition to affecting lipid-induced autoimmunity, the spleen may play a role in the development of NAFLD. As was observed by Oishi and Inoue previously, the present study showed that hepatic lipid accumulation was significantly increased in splenectomized rats. Oishi *et al.*, speculated that splenectomy decreased the liver fatty acid metabolism, thus increasing liver TG content, though no specific mechanism was described.[10] In support of this, Inoue *et al.*, demonstrated that splenectomy suppressed the expression of sterol regulatory element binding protein-1c (SREBP-1c) and carnitine palmitoyltransferase I (CPT1), which are known to play a role in metabolism synthesis of TGs.[10,30,31] Another possible explanation, however, was that the removal of the spleen, the largest lymphoid organ, impacts immune function, resulting in compensation by the liver.[32] For example, Kuppfer cells (KCs), which are specialized macrophages found in the liver, have been shown to increase following splenectomy.[33] It is thought that these KCs may compensate for the loss of immune function associated with splenectomy.[34] Indeed, here, the continuous activation of KCs could induce constitutive proinflammatory cytokine production in the liver, accelerating the development of steatohepatitis.[34] Correspondingly, Huang *et al.*, demonstrated that selective inhibition of KCs with gadolinium chloride could prevent the development of hepatic steatosis.[34]

Here, we suggest that decreased PTEN expression following splenectomy exacerbates NAFLD, induced

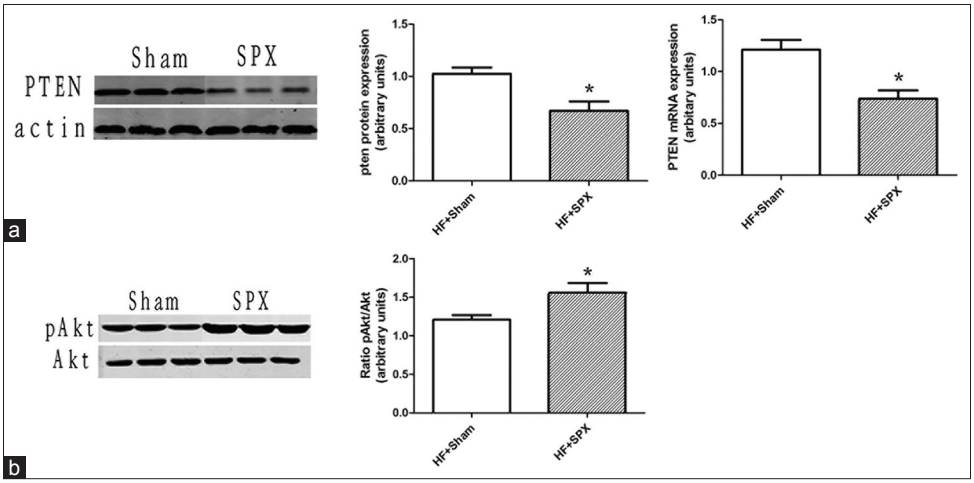

Figure 4: Splenectomy reduces PTEN expression, increasing Akt activity in rats fed a high-fat diet. (a) PTEN expression was determined at the protein level by western blot analysis (left and middle panel) and the mRNA level by RT-PCR (right panel). (b) Akt and pAkt levels were determined by western blot analysis. ND, normal diet; HF, high-fat diet; Sham, sham operation; SPX, splenectomy. $^{*}P < 0.05$ compared with rats having undergone sham operation

by a high-fat diet, in rats. Through its phosphatase activity, PTEN terminates phosphoinositide 3-kinase (PI3K)-propagated signaling by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) to PIP_2, thus acting as an important negative regulator of the PI3K/Akt signal pathway. The PI3K/Akt pathway plays a critical role in nutrient metabolism, cell growth and proliferation, and apoptosis. Previous studies have shown that liver-specific PTEN knockout (KO) mice develop a severe NASH.[12,13] Consistent with this, PTEN expression is decreased in patients and animal models of NAFLD.[18] Furthermore, *in vitro* studies have shown an accumulation of intracellular TGs in HepG2 cells following downregulation of PTEN.[15,18] Furthermore, PTEN mutations/deletions have been linked to ethanol-induced liver injury, viral hepatitis, and liver malignancies,[35] suggesting a critical role for PTEN in the physiopathologic progression of liver disease. PTEN may, thus, make a good potential target for the development of novel therapeutics aimed at treating liver disease.

The spleen has a close anatomical proximity to the liver, and bioactive compounds produced by the spleen, such as cytokines, can directly access the liver via the portal vein.[36] Indeed, splenectomy significantly delays the progression of chemically induced liver fibrosis in rats, partially because of the loss of spleen-derived transforming growth factor-beta1.[36] A potential mechanism whereby the spleen might affect the progression of NAFLD may involve spleen-derived interleukin (IL)-10, as proposed by Goroh *et al*.,[37] They found that: (1). Obesity significantly decreased the expression of splenic IL-10; (2). Splenectomy reduced serum IL-10 levels to a greater extent than other cytokines, inducing lipid accumulation and inflammatory responses in the liver; (3). Exogenous addition of IL-10 counteracted the effect of splenectomy; (4). Splenectomy had little effect on the IL-10 KO mice.[37] This work suggests that the anti-inflammatory activity of spleen-derived IL-10 may play a key role in affecting the development of NAFLD. There are still some limitations in this study. Although we found that splenectomy worsened lipid profile and altered the expression of PTEN, their underlying molecular mechanisms have not been further investigated. Future studies are needed to fully elucidate the molecular mechanism of spleen-mediated regulation of lipid metabolism and its role in the development of NAFLD.

In summary, our study demonstrated that splenectomy increased serum lipids, except TG and HDL, and significantly accelerated hepatic steatosis. In addition, splenectomy resulted in suppression of PTEN expression and, correspondingly, a high ratio of pAkt/Akt in the livers. These data suggest that the spleen may play a role in the development of NAFLD, through modulation of hepatic PTEN expression.

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