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.

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.[19]

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), 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 hematoxylin 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, Beijing, 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:
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 \((P < 0.05, \text{Figure } 2)\). 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 \((all \ P < 0.05, \text{Figure } 2)\).

Effect of splenectomy on hepatic PTEN expression

Several studies have suggested a role for PTEN in the development of NASH. 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)          | 378.7±18.48     | 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      | 14.94±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).

**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. Patients with myeloproliferative diseases associated with hypersplenism, such as polycythemia vera and myelofibrosis, demonstrate reduced TC, HDL and some apolipoproteins. A study of World War II veterans, who underwent splenectomy, demonstrated a high incidence of acute myocardial infarction (MI). 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. 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. Interestingly, compared with the total splenectomy, partial splenectomy and hemisplenectomy have been shown to result...
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.\(^{[29]}\)

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 \textit{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 \textit{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.\(^{[52]}\) For example, Kupffer 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 \textit{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...
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)-triphosphate (PIP₃) to PIP₂, 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.[15,16] 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.[16] 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.[16] 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.

REFERENCES

1. Law K, Brunt EM. Nonalcoholic fatty liver disease. Clin Liver Dis 2010;14:591-604.
2. Adams LA, Angulo P. Recent concepts in non-alcoholic fatty liver disease. Diabet Med 2005;22:1129-33.
3. Vuppalanchi R, Chalasani N. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: Selected practical issues in their evaluation and management. Hepatology 2009;49:306-17.
4. Day CP, James OF. Steatohepatitis: A tale of two "hits"? Gastroenterology 1998;114:842-4.
5. Berson A, De Beco V, Letteron P, Robin MA, Moreau C, El Kahwaji J, et al. Steatohepatitis-inducing drugs cause mitochondrial dysfunction and lipid peroxidation in rat hepatocytes. Gastroenterology 1998;114:764-474.
6. Petroianu A, Veloso DF, Costa GR, Alberti I.R. Effects of splenic surgeries on lipidogram of rats. Rev Assoc Med Bras 2006;52:56-9.
7. Paulo IC, Paulo DN, Kalil M, Guerra AJ, Guerzet EA, Silva AL. Effects of two types of diet on plasma lipids in rats submitted to splenic surgery. Rev Assoc Med Bras 2007;53:171-7.
8. Paulo DN, Paulo IC, Morais AA, Kalil M, Guerra AJ, Colnago GL, et al. Is splenectomy a dyslipidemic intervention? Experimental response of serum lipids to different diets and operations. Microsurgery 2009;29:154-60.
9. Inoue M, Gotoh K, Seike M, Masaki T, Oribe J, Honda K, et al. Involvement of remnant spleen volume on the progression of steatohepatitis in diet-induced obese rats after a splenectomy. Hepatol Res 2012;42:203-12.
10. Oishi T, Terai S, Iwamoto T, Takami T, Yamamoto N, Sakaia I. Splenectomy reduces fibrosis and preneoplastic lesions with increased triglycerides and essential fatty acids in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet. Hepatol Res 2011;41:463-74.
11. Inoue M, Gotoh K, Seike M, Masaki T, Honda K, Kakuma T, et al. Role of the spleen in the development of steatohepatitis in high-fat-diet-induced obese rats. Exp Biol Med (Maywood) 2012;237:461-70.
12. Horie Y, Suzuki A, Kataoka E, Sasaki T, Hamada K, Sasaki J, et al. Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas. J Clin Invest 2004;113:1774-83.
13. Stiles B, Wang Y, Stahl A, Bassilain S, Lee WP, Kim YJ, et al. Liver-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity [corrected]. Proc Natl Acad Sci U S A 2004;101:2082-7.
14. Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 1997;15:356-62.
15. Vinciguerra M, Foti M. PTEN and SHIP2 phosphoinositide phosphatases as negative regulators of insulin signaling. Arch Physiol Biochem 2006;112:89-104.
16. Peyrou M, Bourgoin L, Foti M. PTEN in non-alcoholic fatty liver disease/ non-alcoholic steatohepatitis and cancer. Dig Dis 2010;28:236-46.
17. Matsuda S, Kobayashi M, Kitagishi Y. Roles for PI3K/AKT/PTEN Pathway in Cell Signaling of Nonalcoholic Fatty Liver Disease. ISRN Endocrinol 2013;2013:472432.
18. Vinciguerra M, Veyrat-Dure露 b c, Moukid ML, Rubbia-Brandt L, Rohner-Jeanrenaud F, Foti M. PTEN down-regulation by unsaturated fatty acids triggers hepatic steatosis via an NF-kappaBp65/ mTOR-dependent mechanism. Gastroenterology 2008;134:268-80.
19. Savard C, Tartaglione EV, Kuver R, Haigh WG, Farrell GC, Subramanian S, et al. Synergistic interaction of dietary cholesterol and...
Wang, et al.

and dietary fat in inducing experimental steatohepatitis. Hepatology 2013;57:81-92.

20. Asai K, Kuzuya M, Naito M, Funaki C, Kuzuya F. Effects of splenectomy on serum lipids and experimental atherosclerosis. Angiology 1988;39:497-509.

21. Folch J, Lees M, Sloane SG. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497-509.

22. Schmidt HH, Wagner S, Manns M. The spleen as a storage pool in lipid metabolism. Am J Gastroenterol 1997;92:1072.

23. Gilbert HS, Ginsberg H. Hypocholesterolemia as a manifestation of disease activity in chronic myelocytic leukemia. Cancer 1983;51:1428-33.

24. Le NA, Gibson JC, Rubinstein A, Grabowski GA, Ginsberg HN. Abnormalities in lipoprotein metabolism in Gaucher type 1 disease. Metabolism 1988;37:240-5.

25. Simões FC, Marques RG, Diestel CF, Caetano CE, Dinis AP, Horst NL, et al. Lipidic profile among rats submitted to total splenectomy isolated or combined with splenic autotransplant. Acta Cir Bras 2007;22 Suppl 1:46-51.

26. Petroianu A, Veloso DF, Alberti LR, de Souza Vasconcellos L. Plasma lipid alterations after total splenectomy, subtotal splenectomy and splenic auto-implants in rats. J Gastroenterol Hepatol 2008;23:e221-4.

27. Akan AA, Sengül N, Simşek S, Demirer S. The effects of splenectomy and splenic auto-transplantation on plasma lipid levels. J Invest Surg 2008;21:369-72.

28. Goldfarb AW, Rachmilewitz EA, Eisenberg S. Abnormal low and high density lipoproteins in homozygous beta-thalassemia. Br J Haematol 1991;79:481-6.

29. Matsuura E, Atzeni F, Sarzi-Puttini P, Turiel L, Lopez LR, Nurmohamed MT. Is atherosclerosis an autoimmune disease? BMC Med 2014;12:47.

30. Inoue M, Gotoh K, Seike M, Masaki T, Honda K, Kakuma T, et al. Role of the spleen in the development of steatohepatitis in high-fat-diet-induced obese rats. Exp Biol Med (Maywood) 2012;237:461-70.

31. Baykal A, Aydin C, Hascelik G, Ayhan A, Korkmaz A, Sayek I. Experimental study of the effects of splenectomy and partial splenectomy on bacterial translocation. J Trauma 1999;46:1096-9.

32. Shih-Ching K, Choudhry Ma, Matsutani T, Schwach MG, Rue JW, Bland KI, Chaudry H. Spleen differentially influences immune responses in various tissue compartments of the body. Cytokine 2004;28:101-8.

33. Huang W, Metlakunta A, Dedousis N, Zhang P, Sipula I, Dube JJ, et al. Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. Diabetes 2010;59:347-57.

34. Inoue M, Gotoh K, Seike M, Masaki T, Honda K, Kakuma T, et al. Role of the spleen volume on the progression of steatohepatitis in diet-induced obese rats after a splenectomy. Hepatol Res 2012;42:203-12.

Source of Support: Guangdong Provincial Science and Technology Plan Projects (NO.2013B060300032), Conflict of Interest: None of the authors have a potential conflict of interest or the appearance of a conflict of interest with regard to the work.