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Dietary Phosphatidylinositol Prevents the Development of Nonalcoholic Fatty Liver Disease in Zucker (*fa*/*fa*) Rats

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Recent studies have shown that dietary phospholipids, especially phosphatidylcholine and phosphatidylserine, have various beneficial biological effects. However, there are not enough data concerning the physiological function of dietary phosphatidylinositol (PI). The metabolic syndrome, a cluster of metabolic abnormalities such as dyslipidemia, diabetes mellitus, and hypertension, is a widespread and increasingly prevalent disease in industrialized countries. Nonalcoholic fatty liver disease (NAFLD) is often associated with features of the metabolic syndrome. NAFLD describes the spectrum of liver damage ranging from hepatic steatosis to steatohepatitis, liver fibrosis, and cirrhosis, and it is emerging as the most common liver disease worldwide. The present study examined whether dietary PI protects Zucker (*fa*/*fa*) rats from the metabolic syndrome. For 4 weeks, rats were fed semisynthetic diets containing either 7% soybean oil or 5% soybean oil plus 2% PI. Dietary PI markedly prevented the development of hepatomegaly and hepatic steatosis and lowered hepatic injury markers in serum. Additionally, hyperinsulinemia was relieved by the feeding of dietary PI in Zucker rats. These effects were attributable to an increase in serum adiponectin, enhancement of fatty acid β-oxidation, and suppression of mRNA expression of inflammatory genes in the liver. This is the first report that dietary PI increases serum adiponectin level and prevents the development of NAFLD in a rat model of the metabolic syndrome.

KEYWORDS: Adiponectin; metabolic syndrome; nonalcoholic fatty liver disease; phosphatidylinositol; Zucker (*fa*/*fa*) rats

INTRODUCTION

The metabolic syndrome, a cluster of metabolic abnormalities such as hyperlipidemia, diabetes mellitus, and hypertension, is a widespread and increasingly prevalent disease in industrialized countries and contributes to the increase in cardiovascular morbidity and mortality (*1, 2*). Nonalcoholic fatty liver disease (NAFLD) is often associated with features of the metabolic syndrome and is emerging as the most common liver disease worldwide (*3–6*). NAFLD is the preferred term to describe the spectrum of liver damage ranging from hepatic steatosis to steatohepatitis, liver fibrosis, and cirrhosis. Most liver-related morbidity and mortality are associated with the development of cirrhosis. Cirrhosis is most likely to occur in individuals who have progressed from hepatic steatosis to steatohepatitis. Although the processes through which steatohepatitis evolves from hepatic steatosis are not fully understood, it is necessary to develop effective therapies for the treatment of NAFLD and to discover nutrients that will reduce the risk of NAFLD. Zucker (*fa*/*fa*) rats have hyperphagia, because they have a missense mutation on the leptin receptor gene, and develop a syndrome with multiple metabolic and hormonal disorders including NAFLD that shares many features with the human metabolic syndrome (*7–9*).

Diet, especially dietary fat, contributes to the development and prevention of NAFLD (*10–12*). Although triglycerides make up the majority of dietary fat, phospholipids compose about 3−8% of the daily intake of total dietary fats (*13, 14*). Growing evidence indicates that dietary phospholipids, especially phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), have beneficial effects compared with dietary triglycerides. For example, dietary PC, PE, and PS have been reported to have lipid-lowering effects and to improve brain function, respectively (*15–18*). Phosphatidylinositol (PI) is a

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Table 1. Composition (Grams per Kilogram) of Experimental Diets

ingredient	TG diet	PI diet
casein	200	200
corn starch	150	150
cellulose	50	50
mineral mixture (AIN 76)	35	35
vitamin mixture (AIN 76)	10	10
DL-methionine	3	3
choline bitartrate	2	2
soybean oil	70	50
soybean PI[a]	0	20
sucrose	480	480

[a] Contained 81.3% PI, 14.2% PC, 4.5% PE.

Table 2. Fatty Acid Composition (Weight Percent) of Experimental Diets

fatty acid	TG diet[a]	PI diet[b]
14:0	0.4	0.4
16:0	10.0	11.4
16:1	0.3	0.2
18:0	1.3	1.7
18:1	18.1	16.3
18:2n-6	61.6	61.4
18:3n-3	7.0	7.2

[a] Contained 7% soybean oil as dietary fat. [b] Contained 5% soybean oil + 2% soybean PI as dietary fats.

minor component of dietary phospholipids and is found in legumes and seeds such as soybeans, peanuts, rapeseeds, and sunflower seeds (*19*). Although it has been known for more than 20 years that PI in biological membranes plays key roles in mediating cellular responses to external stimuli (*20, 21*), the physiological function of dietary PI remains poorly understood. In the present study, we investigated the effect of dietary PI on the development of NAFLD in Zucker (*fa/fa*) rats.

MATERIALS AND METHODS

Animals and Diets. All aspects of the experiment were conducted according to the guidelines provided by the ethical committee of experimental animal care at Saga University. Male Zucker (*fa/fa*) rats aged 5 weeks were purchased from Japan SLC (Shizuoka, Japan). The rats were housed individually in metal cages in a temperature-controlled room (24 °C) under a 12 h light/dark cycle. After a 1 week adaptation period, the rats were assigned to two groups (six rats each) that were fed one of two diets: a semisynthetic diet supplemented with 7% soybean oil (TG group) or a semisynthetic diet supplemented with 5% soybean oil plus 2% soybean PI (PI group). The basal semisynthetic diets were prepared according to recommendations of the AIN-76 (*22*). Soybean PI was prepared from soybean phospholipids by enzymatic methods utilizing the phospholipase B having a poor hydrolytic activity on PI. The composition of the semisynthetic diets and the purity of soybean PI are given in **Table 1**. The fatty acid compositions of the TG and PI diets are shown in **Table 2**. The rats consumed the diets for 4 weeks.

Measurement of Serum Parameters. At the end of the feeding period, the rats were sacrificed by aortic exsanguination under diethyl ether anesthesia after a 9 h starvation period. White adipose tissue (WAT) and livers were excised immediately, and serum was separated from the blood. Serum triglyceride and cholesterol levels were measured using commercial enzyme assay kits (Wako Pure Chemicals, Tokyo, Japan). Serum adiponectin (MW = 26839.75) and insulin (MW = 5798.77) levels were measured using commercial rat ELISA kits (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan; Shibayagi Co. Ltd., Gunma, Japan, respectively). Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum were measured using commercial enzyme assay kits (Wako Pure Chemicals, Tokyo, Japan).

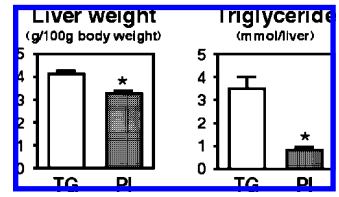

Figure 1. Effect of dietary PI on the relative liver weight and hepatic triglyceride level in Zucker (*fa/fa*) rats. Rats were fed TG diet or PI diet for 4 weeks. Values are expressed as mean ± standard error of six rats. See **Table 1** for composition of diets. Asterisk shows significant difference at $P < 0.05$.

Measurement of Triglyceride and Cholesterol Levels in the Liver. Liver lipids were extracted according to the method of Folch et al. (*23*), and the concentrations of triglyceride and cholesterol were measured by using the methods of Fletcher (*24*) and Sperry and Webb (*25*), respectively.

Preparation of Hepatic Subcellular Fractions. A piece of liver was homogenized in 6 volumes of a 0.25 M sucrose solution that contained 1 mM EDTA in a 10 mM Tris-HCl buffer (pH 7.4). After the nuclei fraction was precipitated, the supernatant was centrifuged at 10000*g* for 10 min at 4 °C to obtain the mitochondria fraction. The resulting supernatant was recentrifuged at 125000*g* for 60 min to precipitate microsomes, and the remaining supernatant was used as the cytosol fraction. The protein concentration was determined according to the method of Lowry et al. (*26*), with bovine serum albumin used as the standard.

Assays of Hepatic Enzyme Activity. The enzyme activities of phosphatidate phosphohydrolase (PAP) in the hepatic microsome fraction (*27*), fatty acid synthase (FAS) in the hepatic cytosol fraction (*28*), and carnitine palmitoyltransferase (CPT) in the hepatic mitochondria fraction (*29*) were determined as described elsewhere.

Analysis of mRNA Expression. Total RNA was extracted from 50 mg of liver, using an RNeasy Lipid Tissue Mini Kit (Qiagen, Tokyo, Japan). A TaqMan Universal PCR Master Mix (Applied Biosystems, Tokyo, Japan); Assay-on-Demand, Gene Expression Products (Rn99999017_m1 for tumor necrosis factor-alpha (TNF-α), Rn00580555_m1 for monocyte chemoattractant protein-1 (MCP-1), Hs99999901_s1 for 18S RNA, Applied Biosystems), was used for the quantitative real-time RT-PCR analysis of TNF-α, MCP-1, and 18S RNA expression in the liver. The amplification was performed with a real-time PCR system (ABI Prism 7000 Sequence Detection System; Applied Biosystems). Results were quantified with a comparative method and were expressed as a relative value after normalization to the 18S RNA expression.

Statistical Analysis. All values are expressed as mean ± SEM. The significance of differences between means for two groups was determined by Student's *t* test. Differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

The two groups of rats did not differ in initial body weight (TG group, 171 ± 3; PI group, 170 ± 2 g), final body weight (TG group, 328 ± 3; PI group, 320 ± 3 g), food intake (TG group, 600 ± 6; PI group, 599 ± 5 g), or total WAT weight (TG group, 18.7 ± 0.4; PI group, 19.5 ± 0.1 g/100 g of body weight). In contrast, the relative liver weight and hepatic triglyceride concentration differed between rats fed the TG and PI diets (**Figure 1**). After a 4 week feeding period, Zucker (*fa/fa*) rats fed the TG diet had severe hepatic steatosis. The relative liver weight was 21% less in PI-fed rats, and this was associated with a marked reduction (77%) in the triglyceride accumulation in the liver. Additionally, hepatic cholesterol level was markedly decreased (by 40%) in PI-fed rats (TG group, 99.7 ± 4.4 μmol/liver; PI group, 59.4 ± 1.4 μmol/liver, $P < 0.05$), a result that is in agreement with previous studies showing that PI administration promotes cholesterol transport and excretion in

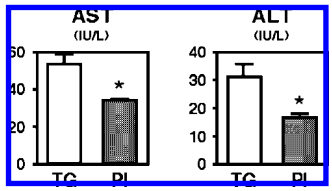

Figure 2. Effect of dietary PI on hepatic injury marker activities in serum of Zucker (*fa*/*fa*) rats. Rats were fed TG diet or PI diet for 4 weeks. Values are expressed as mean ± standard error of six rats. See **Table 1** for composition of diets. Asterisk shows significant difference at $P < 0.05$.

Table 3. Effect of Dietary PI on Activities (Nanomoles per Minutes per Milligram of Protein) of Hepatic Triglyceride Metabolism Related Enzymes in Zucker (*fa*/*fa*) Rats[a]

	TG group	PI group
PAP	20.8 ± 0.9	21.5 ± 1.4
FAS	17.2 ± 0.3	16.3 ± 0.7
CPT	4.87 ± 0.23	5.66 ± 0.24[b]

[a] Values are expressed as mean ± standard error of six rats. [b] Significant difference at $P < 0.05$.

rabbits (*30, 31*). The extent and the direction (increase or decrease) of changes in liver lipoprotein synthesis and secretion during the development of NAFLD have been controversial (*4, 5*). Serum cholesterol level significantly decreased (TG group, 4.93 ± 0.23 mmol/L; PI group, 4.01 ± 0.15 mmol/L, $P < 0.05$), and serum triglyceride level, not significantly, tended to increase (TG group, 2.55 ± 0.34 mmol/L; PI group, 3.94 ± 0.77 mmol/L, $P = 0.130$) in the PI group as compared with the TG group. Consistent with the alleviation of hepatomegaly and hepatic steatosis by the PI diet, however, the activities of hepatic injury markers such as AST and ALT were markedly decreased (by 37 and 47%, respectively) in the serum of PI-fed rats compared with TG-fed rats (**Figure 2**). Although we did not perform a hepatic histological evaluation, these data suggest that dietary PI protects Zucker (*fa*/*fa*) rats from the development of NAFLD.

To examine further the effect of dietary PI on the liver, hepatic enzymes related to triglyceride metabolism were analyzed (**Table 3**). Activities of PAP and FAS, which are key enzymes in the regulation of triglyceride and fatty acid de novo synthesis, did not differ between the groups. However, the activity of CPT, a key enzyme of fatty acid β-oxidation, was significantly greater in the PI group as compared with the TG group. Although the mechanisms responsible for the development of NAFLD are unclear, it has been suggested that hepatic steatosis results from accelerated mobilization from expanded visceral fat stores and their deposition in the liver as well as decreased hepatic fatty acid β-oxidation (*4, 5*). Thus, we suggest that the prevention of NAFLD by dietary PI is partially attributable to the enhancement of CPT activity in the liver.

Insulin resistance is the essential first pathological step in the development of NAFLD (*32–34*). In fact, hepatic steatosis is now proposed as a feature of the insulin resistance syndrome along with type 2 diabetes, visceral obesity, hyperlipidemia, and hypertension (*32–34*). After a 4 week feeding period, Zucker (*fa*/*fa*) rats given the TG diet had severe hyperinsulinemia. As shown in **Figure 3**, the serum insulin level tended to decrease (by 35%) in the PI group as compared with the TG group. Recently, it has been recognized that adipose tissue not only stores excess energy in the form of fat but also secretes physiologically active substances called adipocytokines (*35*). Among those, adiponectin is one of the most abundant secretory proteins from adipose tissue in rodents and humans (*36, 37*). Because several studies indicate that adiponectin can lead to

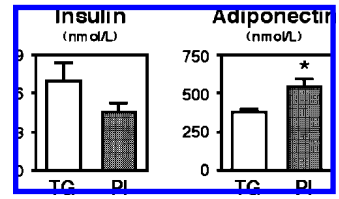

Figure 3. Effect of dietary PI on serum insulin and adiponectin levels in Zucker (*fa*/*fa*) rats. Rats were fed TG diet or PI diet for 4 weeks. Values are expressed as mean ± standard error of six rats. See **Table 1** for composition of diets. Asterisk shows significant difference at $P < 0.05$.

enhanced insulin action in vitro and in vivo, it is strongly suggested that adiponectin plays a protective role against insulin resistance (*38–40*). In the present study, the serum adiponectin level was markedly increased (by 43%) in the PI group as compared with the TG group (**Figure 3**). These results suggest that dietary PI improves insulin resistance through increased serum adiponectin level. Given the finding of a previous study that pioglitazone, one of the insulin-sensitizing drugs, relieves insulin resistance with increasing serum adiponectin levels in Zucker rats (*41*), we suppose that dietary PI might act as an insulin sensitizer. Moreover, several papers indicate that adiponectin enhances fatty acid oxidation by activating AMP-activated protein kinase and PPAR-α in the liver and muscle (*42, 43*). Thus, we consider that the increase in serum adiponectin may contribute to enhancement of hepatic fatty acid β-oxidation in PI-fed rats. In addition, a recent study demonstrated that PI administration increased the levels of high-density lipoprotein (HDL)-cholesterol in humans (*44*). Because adiponectin increased HDL assembly in human hepatocytes (*45*), dietary PI might be a functional food that increases HDL-cholesterol levels through the enhancement of adiponectin production.

The pathogenesis of steatohepatitis, the more advanced form of NAFLD, has yet to be clearly defined, but the recent major theory is the "two-hit" hypothesis (*46, 47*). The first hit is the triglyceride accumulation within the liver. It was proposed that lipid-laden hepatocytes are more susceptible to a second hit, that is, injury by oxidative stress and inflammatory cytokines, such as TNF-α. In addition, lipid peroxidation products trigger cytokine production within the liver, and this accelerates TNF-α-mediated liver injury. In fact, an overexpression of TNF-α mRNA is found in the liver of nonalcoholic steatohepatitis patients (*48*). Moreover, it has been recognized that MCP-1, a member of the CC chemokine family, induces inflammatory responses through recruiting inflammatory cells and is up-regulated by inflammatory stimuli such as TNF-α (*49, 50*). Interestingly, recent findings also indicate that transgenic mice expressing MCP-1 exhibit insulin resistance and hepatic steatosis, whereas a disappearance of MCP-1 in knockout mice and an acute inhibition of MCP-1 by expression of a dominant-negative mutant in mice resulted in improvement of insulin resistance and hepatic steatosis (*51*). In the present study, as shown in **Figure 4**, mRNA expression of TNF-α tended to decrease (by 58%) and MCP-1 mRNA expression was drastically decreased (by 93%) in the liver of PI-fed rats compared with TG-fed rats. Because adiponectin has protective effects against both inflammation (*52*) and fibrosis (*53*), we consider that the increase in serum adiponectin by dietary PI may contribute to the prevention of development and progression of NAFLD in Zucker (*fa*/*fa*) rats. Moreover, the present data lead us to speculate that adiponectin and MCP-1 suppress each other's production and also antagonize each other's action in their target tissue, given that previous studies found that serum

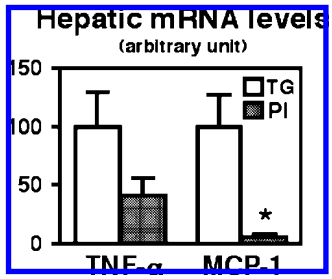

Figure 4. Effect of dietary PI on mRNA expressions of inflammatory genes in the liver of Zucker (*fa/fa*) rats. Rats were fed TG diet or PI diet for 4 weeks. Values are expressed as mean ± standard error of six rats. See **Table 1** for composition of diets. Asterisk shows significant difference at $P < 0.05$.

adiponectin was significantly increased in MCP-1 knockout mice compared with wild-type controls (*51*).

In conclusion, our present study is the first report that dietary PI increases serum adiponectin level and prevents the development of NAFLD in a rat model of the metabolic syndrome. Although fatty acid compositions of dietary fats have been recognized as contributing factors for the development and prevention of NAFLD, there were no significant differences of fatty acid composition between the TG and PI diets (**Table 2**). Therefore, we supposed that the beneficial effects demonstrated in this study were attributable to the physiological functions of soybean PI itself. However, further studies are necessary to evaluate the dose dependency and the lowest effective concentration of dietary PI or its constituent base inositol. Additionally, given that dietary PC and PE also have lipid-lowering effects, comparison concerning physiological effects on the development and prevention of metabolic syndrome among phospholipids, such as PC, PE, and PI, or their constituent bases would be of great interest for future study.

ABBREVIATIONS USED

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPT, carnitine palmitoyltransferase; FAS, fatty acid synthase; MCP-1, monocyte chemoattractant protein-1; NAFLD, nonalcoholic fatty liver disease; PAP, phosphatidate phosphohydrolase; PI, phosphatidylinositol; TG, triglyceride; TNF-α, tumor necrosis factor-alpha; WAT, white adipose tissue.

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