ORIGINAL

Palatinose and oleic acid act together to prevent pancreatic islet disruption in nondiabetic obese Zucker rats

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Abstract : We showed previously that 8-wk consumption of a diet containing palatinose (P, a slowly-absorbed sucrose analogue) and oleic acid (O) ameliorates but a diet containing sucrose (S) and linoleic acid (L) aggravates metabolic abnormalities in Zucker fatty (*fa/fa*) rats. In this study, we aimed to identify early changes in metabolism in rats induced by certain combinations of carbohydrates and fatty acids. Specifically, male Zucker fatty rats were fed an isocaloric diet containing various combinations of carbohydrates (P ; S) and fatty acids (O ; L). After 4 wk, no significant differences in body weight, visceral fat mass, plasma parameters (glucose, insulin, lipids, and adipokines), hepatic adiposity and gene expression, and adipose inflammation were observed between dietary groups. In contrast, pancreatic islets of palatinose-fed (PO and PL) rats were smaller and less fibrotic than sucrose-fed (SO and SL) rats. The abnormal α-cell distribution and sporadic staining of active caspase-3 common to islets of linoleic-acid-fed rats were not observed in oleic-acid-fed (PO and SO) rats. Accordingly, progressive β-cell loss was seen in SL rats, but not in PO rats. These findings suggest that pancreatic islets may be initial sites that translate the effects of different combinations of dietary carbohydrates and fats into metabolic changes. J. Med. Invest. 55 : 183-195, August, 2008

Keywords **:** *Zucker fatty (fa/fa) rats, isomaltulose (palatinose), sucrose, oleic acid, linoleic acid, pancreatic islet*

List of Abbreviations : The abbreviations used are : ANOVA, analysis of variance ; Caspase-3, cysteinyl aspartate-specific protease-3 ; CLS, crown-like structure ; DAB, diaminobenzidine ; FFA, free fatty acid ; HPETE, hydroperoxyeicosatetraenoic acid ; LXRα, liver X receptor α ; MUFA, monounsaturated fatty acid ; PEPCK, phosphoenolpyruvate carboxykinase ; PL, palatinose and linoleic acid ; PO, palatinose and oleic acid ; PPARα, peroxysome proliferator-activated receptor α ; PUFA, polyunsaturated fatty acid ; SFA, saturated fatty acid ; SL, sucrose and linoleic acid ; SO, sucrose and oleic acid ; SREBP, sterol regulatory-element binding protein ; TG, triglyceride ; TNFα, tumor necrosis factor α ; WAT, white adipose tissue.

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INTRODUCTION

Visceral obesity is the most prevalent manifestation of a metabolic syndrome that associates closely with insulin resistance, type 2 diabetes, and cardiovascular disease. Although complex interactions between many genetic and environmental factors contribute to the development of obesity, diet is considered the single most important etiological factor. Human and animal studies have shown that excess intake of simple sugars and fats is the principal lifestyle-related cause of both obesity and obesity-related metabolic disorders that contribute to the metabolic syndrome (1, 2).

It has been suggested that specific types of dietary carbohydrates and fats influence the risk of developing diet-induced metabolic disorders. Rapidly digestible (i.e., high glycemic) saccharides such as sucrose and fructose that increase blood glucose levels, particularly in the postprandial period, have a consequent effect on insulin and triglyceride levels and have been suggested to increase risks of obesity and insulin resistance (3). In contrast, slowly digestible (i.e., low glycemic) carbohydrates such as oligosaccharides and resistant starches have been shown to prevent postprandial hyperglycemia and hyperinsulinemia (1). Ingestion of palatinose [isomaltulose ; 6-0-(α-0-glucopyranosyl)-D-fructofuranose], a slowly digestible sucrose analog composed of α-1,6-linked glucose and fructose, has been reported to improve diabetic symptoms (4). Epidemiological studies of ingested fats indicate that while insulin resistance and obesity-related diseases worsen with ingestion of saturated fatty acids, these conditions improve with ingestion of polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA) (2). Linoleic and oleic acids are two of the major fatty acids found in dietary fat and plasma triglycerides. Linoleic acid 18 : 2(*n*-6) has been implicated in upregulation of inflammatory responses via its conversion to arachidonic acid 20 : 4(*n*-6), which is a precursor to inflammatory eicosanoids such as prostaglandins and hydroperoxyeicosatetraenoic acids (HPETEs) (5). On the other hand, dietary MUFA [particularly oleic acid 18 : 1(*n*-9)] has been linked to a low prevalence of atherosclerosis, beneficial changes in lipoprotein distribution, and improved pancreatic β-cell secretory function following disruption by chronic hyperglycemia and hyperlipidemia (6-9).

One significant limitation to the interpretation of the aforementioned studies is that none focus on nutrient-nutrient interactions. Diets consist of food mixtures containing complex combinations of interactive and synergistic nutrients, and these combinations and interactions need to be taken into account. We reported previously that a liquid balanced formula (MHN-01/Inslow®) containing palatinose and oleic acid suppressed postprandial hyperglycemia and hyperinsulinemia, reduced visceral fat accumulation, and improved insulin sensitivity in healthy men and Sprague-Dawley rats (10, 11). We also have shown that various symptoms of obesity in Zucker fatty (*fa/fa*) rats were attenuated by long-term (8 wk) consumption of a diet containing palatinose and oleic acid, but aggravated by a diet containing sucrose and linoleic acid (12). These results suggest that dietary carbohydrates and fats could influence glucose and lipid metabolism concertedly *in vivo*. Because the differential effects of these combinations of nutrients were not fully investigated in the foregoing study, our aim in this study was to examine the effects of combinations of dietary carbohydrates (palatinose and sucrose) and fats (oleic acid and linoleic acid) on the metabolic profile in obesity. Our specific aim was to identify the organ in Zucker fatty rats responsible for the initial metabolic alterations induced by experimental diets, a key discovery toward development of a strategic dietary approach for preventing obesity-related metabolic disorders.

MATERIALS AND METHODS

Animals

Twenty male, 11 wk old Zucker fatty (*fa/fa*) rats (Charles River, Kanagawa, Japan) weighing 450-470 g were caged individually in a climate-controlled room (temperature, 23±1°C ; humidity 70 to 75% ; specific pathogen-free) with a 12 h light/dark cycle. Prior to the initiation of our study, the rats were allowed free access to standard rodent powder diet (MF ; Oriental Yeast, Tokyo, Japan) and water.

Diets

The experimental diets were prepared fresh weekly, sealed in air-tight plastic bags, and stored at 4°C to avoid rancidity. The composition of each diet is listed in Table 1. Safflower oil served as the linoleic acid-rich oil (L) ; a mixture of high oleic sunflower oil and perilla oil served as the oleic acid-rich oil (O). The final composition of the diet (g/kg) was 212.8 of total milk protein and casein, 353.2 of pa-

Table 1. Composition of Experimental Diets[1]

	Diet			
	SL	SO	PL	PO
	g/kg diet			
Total milk protein and casein [2]	212.8	212.8	212.8	212.8
Fat [3]	140.0	140.0	140.0	140.0
Carbohydrate	510.7	510.7	510.7	510.7
Indigestible dextrin	63.8	63.8	63.8	63.8
Vitamin and mineral mixture [4]	72.3	72.3	72.3	72.3
Fatty acid composition	%			
palmitic acid 16 : 0	7.3	6.7	7.3	6.7
stearic acid 18 : 0	2.6	4.2	2.6	4.2
oleic acid 18 : 1(n-9)	13.4	72.3	13.4	72.3
linoleic acid 18 : 2(n-6)	76.4	10.7	76.4	10.7
α-linolenic acid 18 : 3(n-3)	0.2	4.4	0.2	4.4
Other fatty acids	-	1.7	-	1.7
Carbohydrate composition	%			
Palatinose	0.0	0.0	70.0	70.0
Sucrose	70.0	70.0	0.0	0.0
Dextrin	30.0	30.0	30.0	30.0
Total energy [5], kJ/g	17.8	17.8	17.8	17.8

[1] All of the diets were identical except for the types of fats and carbohydrates : (SL) sucrose and linoleic acid diet ; (SO) sucrose and oleic acid diet ; (PL) palatinose and linoleic acid diet ; (PO) palatinose and oleic acid diet.
[2] Percentages of total milk protein and casein were 60 and 40, respectively.
[3] The fat component of L or O was derived from safflower oil or high-oleic sunflower oil blended with perilla oil, respectively. Percentages of high-oleic sunflower oil and perilla oil were 90 and 10, respectively.
[4] The weights of vitamins and minerals (per kg of diet) were as follows. Vitamins supplied : 3.2 mg retinyl palmitate, 31.9 μg cholecalciferol, 340.4 mg α-tocopherol, 42.6 μg menadione and phylloquinone, 255.3 mg thiamin HCl, 21.3 mg riboflavin, 89.4 mg nicotinamide, 12.8 mg pyridoxine HCl, 42.6 mg calcium pantothenate, 1.7 g L-ascorbic acid, 825.5 mg choline, 2.1 mg folic acid, 38.3 μg cyanocobalamin and 12.3 μg biotin. Minerals supplied : 3.0 g sodium, 3.4 g calcium, 42.6 mg iron, 3.4 g phosphorus, 1.1 g magnesium, 3.4 g potassium, 2.1 mg copper, 0.4 mg manganese, 42.6 mg zinc, 2.6 g chloride, 148.9 μg selenium, 127.7 μg chromium, 119.1 μg iodine and 123.4 μg molybdenum.
[5] Percentages of energy as fat, carbohydrate and protein were 29.7, 50.3 and 20.0, respectively.

latinose or sucrose, 157.5 of dextrin, 63.8 of indigestible dextrin, 72.3 of vitamin and mineral mixture, and 140.4 of test oil.

Experimental design

Rats were randomly assigned to one of 4 dietary groups, each containing 4 rats, that were fed either a diet rich in sucrose and linoleic acid (SL), sucrose and oleic acid (SO), palatinose and linoleic acid (PL), or palatinose and oleic acid (PO). To eliminate differences in energy states due to altered palatability among these diets, PO-based pair-feeding was performed as previously described (12). Food intake was monitored and the food itself was replaced daily ; body weights were recorded weekly throughout the feeding period.

After four weeks, the rats were deprived food for 24 h and then sacrificed for blood and tissue collection. Blood samples collected from the rats' tail veins were used to determine plasma glucose and insulin levels. The rats were then anesthetized with diethyl ether and blood was withdrawn from the jugular vein for use in all other measurements. To avoid the influences of intestinal contents on these measures (which in a preliminary feeding experiment proved considerable after a 12 h-only fast), blood composition determinations were made after a 24 h fast. Rats were subsequently sacrificed for harvest and weight measurement of liver, visceral fat and pancreatic samples.

The Institutional Animal Care and Oversight Committee approved the experimental protocols of the study, which were carried out according to the guidelines and principles for the care and use of animals at the University of Tokushima.

Plasma glucose, insulin, lipid and adipocytokine concentrations

Plasma glucose was determined using the glucose dehydrogenase method and an Accu-Chek

blood glucose meter (Roche Diagnostics, Tokyo, Japan). Plasma insulin was measured by ELISA (Morinaga, Yokohama, Japan). Plasma triglyceride, cholesterol, and free fatty acid (FFA) levels were determined using Triglyceride-E-, Cholesterol-E-, and NEFA-C- tests (Wako Pure Chemical Industries, Osaka, Japan), respectively. Commercial ELISAs were used to measure plasma adiponectin (Otsuka Seiyaku, Tokyo, Japan) and TNFα concentrations (Biosource International, Camarillo, CA, USA).

Hepatic triglyceride concentration

Hepatic lipids were extracted as described previously (13). Triglyceride levels were measured in lipid extracts resuspended in methanol using a commercial kit (Triglyceride-E-kit, Wako Pure Chemical Industries).

RNA preparation and quantitative RT-PCR

To assess diet-induced changes in gene expression, steady-state mRNA levels from genes involved in glucose and lipid homeostasis were measured in PCR analyses of reverse-transcribed total RNA templates (RT-PCR). Total RNA was isolated from snap-frozen liver samples using the commercially available acid-phenol reagent Trizol (Invitrogen, Carlsbad, CA, USA). First-strand cDNAs were synthesized from 5 μg of total RNA using an oligo-dT primer and M-MLV reverse transcriptase (Invitrogen) at 42°C for 60 min and 95°C for 5 min. Real-time PCR using gene-specific primers and SYBR green dye (SYBR® Premix Ex Taq™ ; TAKARA BIO, Shiga, Japan) were performed in a LightCycler (Roche Diagnostics) according to the manufacturer's instructions. The following primers were used : *PEPCK* [GenBank : AH007109] (*forward*, 5'-AGACAAATCCGAACGCCATT-3' ; *reverse*, 5'-CATCCTGTGGTCTCCACTCT-3') : *glucokinase* [GenBank : J04218] (*forward*, 5'-GTGAGGCACG-AAGACCTAGA-3' ; *reverse*, 5'-CTGTGTCGTTCA-CCATTGCC-3') : *LXRα* [GenBank : NM_031627] (*forward*, 5'-CCTCAAGATGCAGGAGACCA-3' ; *reverse*, 5'-TAGGCTCGGATGACTCCAAC-3') : *SREBP-1* [GenBank : AF286470] (*forward*, 5'-GGAGCCATGGATTGCACATTT-3' ; *reverse*, 5'-TCCTTCCGAAGGTCTCTCCTC-3') : *SREBP-2* [GenBank : XM_216989] (*forward* , 5'-ACTGTCAC-TGGAGTCAGGTT-3' ; *reverse*, 5'-GACCAACAGCT-TCACGAAGA-3') : *PPARα* [GenBank : M88529] (*forward*, 5'-TGTATGAAGCCATCTTCACG-3' ; reverse, 5'-GGCATTGAACTTCATAGCGA-3') : *TNFα* [GenBank : NM_031144] (*forward*, 5'-ATGGATC-TCAAAGACAACCA-3' ; *reverse*, 5'-TCCTGGTAT-GAAATGGCAAA-3'). For each sample, the expression levels of individual genes were normalized relative to constitutively expressed β-*actin* [GenBank : NM_031144] (*forward*, 5'-GTCCCAGTATGCCT-CTGGTCGTAC-3' ; *reverse*, 5'-CCACGCTCGGT-CAGGATCTTCATG-3') in the same sample, and then expressed as a ratio relative to the same gene's expression level in the SL group (arbitrarily set at 1).

Histological and immunohistochemical analyses

Liver, epididymal adipose tissue and pancreatic samples were fixed in 4% buffered paraformaldehyde, embedded in paraffin, sectioned, deparaffinized in xylene, stained with Mayer's hematoxylin and eosin (Wako Pure Chemical Industries) and examined by light microscopy. Masson's trichrome stain (14) was performed to assess islet fibrosis.

For immunohistochemical staining, slides were microwaved for 5 min in 10 mmol/L sodium citrate (pH 6.0) to retrieve antigen. After blocking in 5% BSA for 2 h at room temperature, slides were incubated with primary antibodies as follows : overnight at 4°C for rabbit polyclonal IgG anti-F4/80 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal IgG anti-insulin (Nichirei Bioscience, Tokyo, Japan) ; for 30 min at room temperature for rabbit polyclonal IgG anti-glucagon (Thermo Scientific, CA, USA) ; for 5 h at 4°C for rabbit polyclonal IgG anti-active/cleaved caspase-3 (Imgenex, CA, USA). Slides were subsequently washed in PBS and incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (En-Vision+ System-HRP ; DakoCytomation, Carpinteria, CA, USA) for 1 h at room temperature. The immunoreactivity of each sample was visualized with DAB (Nakalai Tesk, Kyoto, Japan) and samples were counterstained with Mayer's hematoxylin (Wako Pure Chemical Industries).

Cell areas of adipocyte, pancreatic islet, insulin-immunoreactive β-cell areas and intraislet fibrosis areas were measured with masking using Image-Pro Plus® 6.0 Software (Media-Cybernetics, Silver Spring, MD, USA). The frequency of crown-like structure (CLS) formation was calculated from micrographs as follows : [number of F4/80 positive CLS/number of total adipocytes] x100.

Statistical analysis

Results are expressed as mean ± SEM. Statistically significant effects of dietary carbohydrate and fat were determined using two-way ANOVA fol-

lowed by the Tukey-Kramer post hoc test in cases of significant interaction. To test for significant differences in morphological measures of pancreatic islets and epididymal fat cells, we applied the non-parametric Kruskal-Wallis test followed by Scheffe's post hoc test. A P value of <0.05 was considered significant. Statistical tests were performed using StatView 5.0 (SAS, Cary, NC, USA) and Excel-Toukei 2006 (SSRI, Tokyo, Japan).

RESULTS

Body and organ weights and blood chemistry

During the initial 4 wk feeding period, energy intake was similar in all groups (Table 2). Although the four groups of rats did not differ significantly in body weight or visceral fat accumulation, epididymal fat pads of the PL group were significantly heavier than the PO group. Relative liver weights were not affected by the treatments. The diet treatment also did not result in significant changes in plasma concentrations of glucose, insulin, triglyceride, total cholesterol, FFA, adiponectin and TNFα (Table 3).

Liver

At both a gross and histological level, the appearance of liver fattiness was similar in all groups (Fig. 1A), and was matched by an absence of significant differences in hepatic triglyceride levels ($P=0.737$, two-way ANOVA) (Fig. 1B).

Expression of PEPCK (gluconeogenesis), glucokinase (glycolysis), SREBP-1, SREBP-2, LXRα (lipogenic transcription factors) and PPARα (a transcription factor in the nuclear receptor superfamily regulating fatty acid β-oxidation), which are important for hepatic regulation of blood glucose and lipids, was measured by RT-PCR from liver total RNA. As predicted by the constancy of plasma glucose and lipid concentrations, expression levels of these genes also were not affected by changes in diet (Fig. 1C). Although a previous report demonstrated

Table 2. Body and organ weights and energy intake of Zucker fatty rats fed combinations of carbohydrates (C) and fats (F) for four weeks[1]

	Diet				2-Way ANOVA		
					C	F	C × F
Variable	SL	SO	PL	PO	P-values		
Body weight, g	537.6 ± 7.4	539.9 ± 15.8	536.8 ± 7.6	551.3 ± 12.0	0.432	0.720	0.873
Visceral fat, *g/kg BW*	115.4 ± 2.5	111.6 ± 3.8	112.3 ± 4.3	104.3 ± 3.4	0.189	0.136	0.604
Mesenteric fat	36.1 ± 1.6	34.4 ± 1.8	33.9 ± 2.6	32.0 ± 0.4	0.224	0.339	0.948
Epididymal fat	31.0 ± 0.2 [a,b]	31.6 ± 0.8 [a,b]	34.9 ± 1.3 [a]	29.7 ± 1.5 [b]	0.319	0.070	0.025
Retroperitoneal fat	48.4 ± 1.1	45.6 ± 1.7	43.5 ± 1.6	42.7 ± 2.7	0.058	0.341	0.600
Liver, *g/kg BW*	29.1 ± 3.4	25.2 ± 2.2	32.9 ± 1.1	28.8 ± 1.6	0.121	0.098	0.990
Energy intake, *KJ/d*	395.9 ± 10.5	388.4 ± 15.3	401.4 ± 12.0	402.8 ± 13.1	0.455	0.816	0.736

[1] Values are means ± SEM, n=4. Within a row, means marked with unlike superscripts are significantly different, $P<0.05$.

Table 3. Plasma concentrations of glucose, insulin, lipids and adipokines in Zucker fatty rats fed combinations of carbohydrates (C) and fats (F) for four weeks[1]

	Diet				2-Way ANOVA		
					C	F	C × F
Variable	SL	SO	PL	PO	P-values		
Glucose, *mmol/L*	5.7 ± 1.0	4.8 ± 0.2	5.2 ± 0.4	5.0 ± 04	0.796	0.405	0.578
Insulin, *pmol/L*	3010 ± 330	2500 ± 110	3260 ± 340	2980 ± 200	0.187	0.156	0.659
Triglyceride, *mmol/L*	2.17 ± 0.14	2.00 ± 0.20	2.47 ± 0.13	2.63 ± 0.50	0.085	0.941	0.506
Total cholesterol, *mmol/L*	3.41 ± 0.38	3.52 ± 0.34	4.28 ± 0.10	3.67 ± 0.32	0.176	0.507	0.296
Free fatty acid, *mmol/L*	0.89 ± 0.96	0.96 ± 0.08	0.94 ± 0.12	0.91 ± 0.06	0.974	0.852	0.577
Adiponectin, *mg/L*	7.36 ± 0.43	7.57 ± 0.49	7.33 ± 0.24	7.49 ± 0.37	0.924	0.635	0.955
TNFα, *ng/L*	39.79 ± 9.23	26.39 ± 2.19	45.89 ± 14.61	30.26 ± 4.35	0.874	0.120	0.900

[1] Values are means ± SEM, n=4.

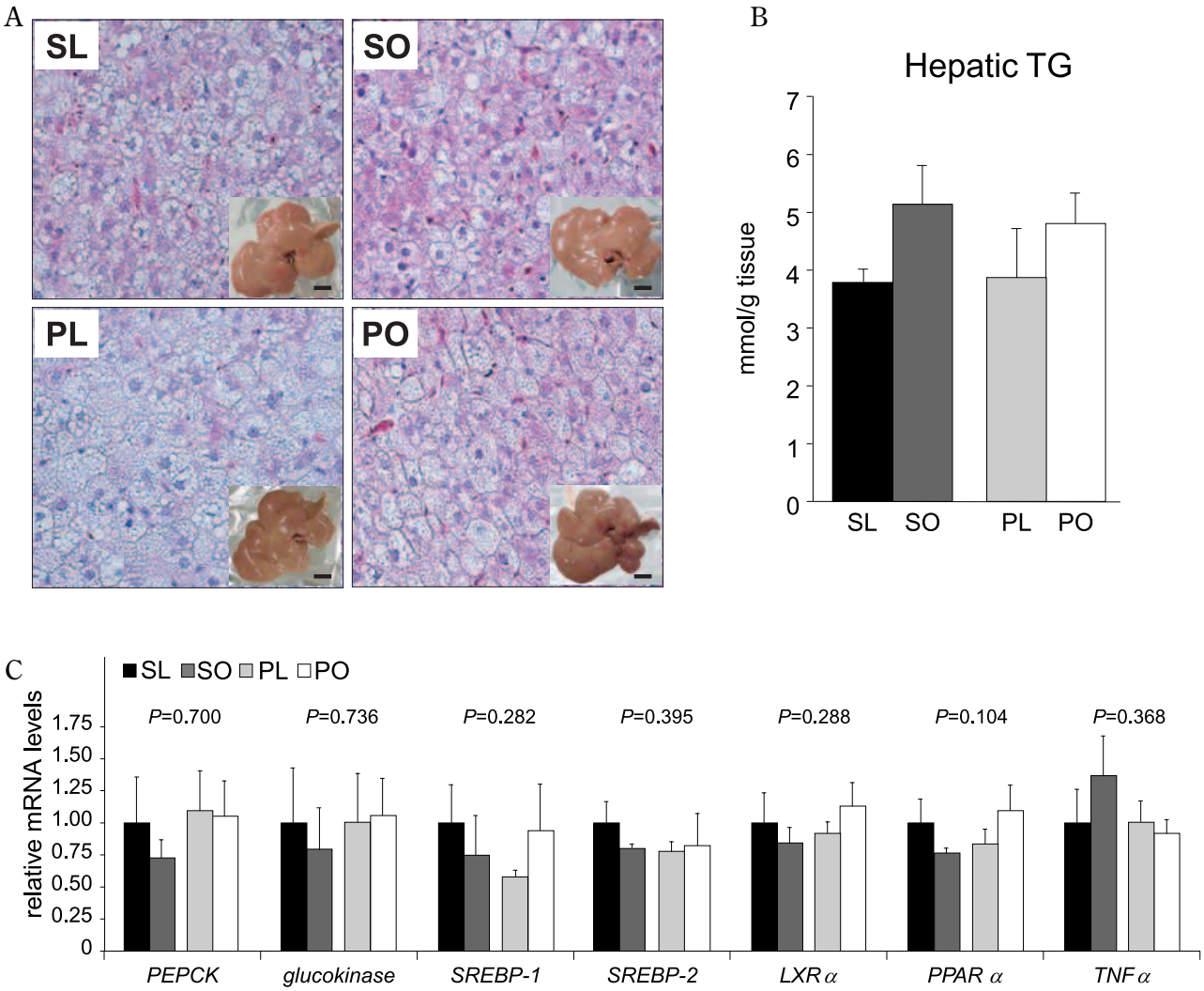

Fig. 1. Hepatic steatosis and gene expression in Zucker fatty rats fed combinations of carbohydrates and fats for four weeks (A) HE-stained paraffin-embedded liver sections (4 μm-thick, original magnification, ×200) and gross appearance of livers (scale bar, 1 cm) from SL, SO, PL, or PO rats. (B) Triglyceride content of liver. (C) Quantitative real-time PCR analysis of mRNA levels from hepatic genes relevant to glucose and lipid metabolism and inflammation (normalized to β-actin). Normalized expression levels from the SL group were set arbitrarily at 1. Values are means ± SEM, n=4 per group. *P* values, by 2-way ANOVA.

that Zucker fatty rats fed the SL and PL diets for 8 weeks showed increased levels of pro-inflammatory gene mRNAs in the liver (12), we observed no significant differences in hepatic TNFα gene expression or plasma TNFα concentrations between these groups in the current study (Table 3).

Adipose tissue

No significant differences in mean adipocyte area were detected between groups ($P=0.096$, Kruskal-Wallis test), indicating that the 4 wk-diet treatment did not affect adipocyte size (Fig. 2A).

White adipose tissue (WAT) inflammation, which is attributed primarily to infiltrating pro-inflammatory macrophages, is an early event in the development of obesity-related metabolic disorders (15, 16). Localization of macrophages to CLS around individual adipocytes occurs during WAT inflammation, and increases in density with increased obesity (17). Three measures indicative of macrophage density were assessed from adipose tissue samples of each diet group : the intensity of F4/80-immunostaining (i.e., macrophages) (Fig. 2B), steady-state levels of TNFα, cyclooxygenase-2 and Emr1 (F4/80) mRNAs (data not shown), and the formation of F 4/80-positive CLS in epididymal WAT ($P=0.384$, two-way ANOVA) (Fig. 2B). We observed no significant differences in any of these parameters between diet groups. These results indicate that four weeks of ingesting these experimental diets had no discernable effect on development of WAT inflammation in Zucker fatty rats.

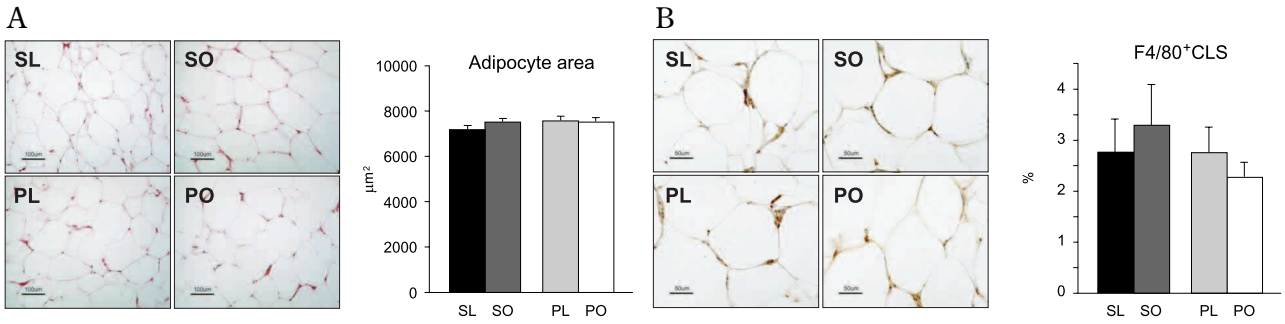

Fig. 2. Adipocyte morphology and WAT macrophage localization in Zucker fatty rats fed combinations of carbohydrates and fats for four weeks
(A) HE-stained paraffin-embedded sections (7 μm-thick, magnification bar=100 μm) and adipocyte area of epididymal WAT from SL, SO, PL, or PO rats. At least 300 adipocytes from each rat were analyzed. (B) Anti-F4/80-immunostained paraffin-embedded sections (7 μm-thick, magnification bar=50 μm) and frequencies of F4/80-positive crown-like structure (CLS) formation of epididymal WAT from SL, SO, PL, or PO rats. Values are means ± SEM, n=4 per group.

PANCREAS

Morphology

In contrast, we observed striking differences in islet morphology in HE-stained pancreatic sections derived from rats in the four diet groups (Fig. 3). After 4 wk of treatment, islets from sucrose-fed (SL and SO) rats were large and irregular-shaped, with some expansion into adjacent exocrine tissue suggestive of islet hypertrophy. Overall, these abnormal, irregularly shaped islets were most abundant in SL rats. The islets from palatinose-fed (PL and PO) rats were smaller than sucrose-fed rats; section from PO rats showed a large number of normal, round or oval islets, whereas some irregularity in shape was apparent in islets from PL rats (Fig. 3A). Mean islet area in the sucrose-fed groups was significantly larger than in the palatinose-fed groups (Fig. 3B); these results were consistent with the features observed in the Zucker fatty rats fed same diets for 8 wk (12). Thus, islet hypertrophy associated with consumption of dietary sucrose, as opposed to palatinose.

When samples were stained for insulin, intense brown, insulin-positive staining of islets was seen most frequently in PO rats relative to the other three groups. In contrast, SL rats maintained islets that showed an attenuated and variable pattern of staining suggestive of progressive β-cell loss (Fig. 3A). Consistent with staining, the mean pro-

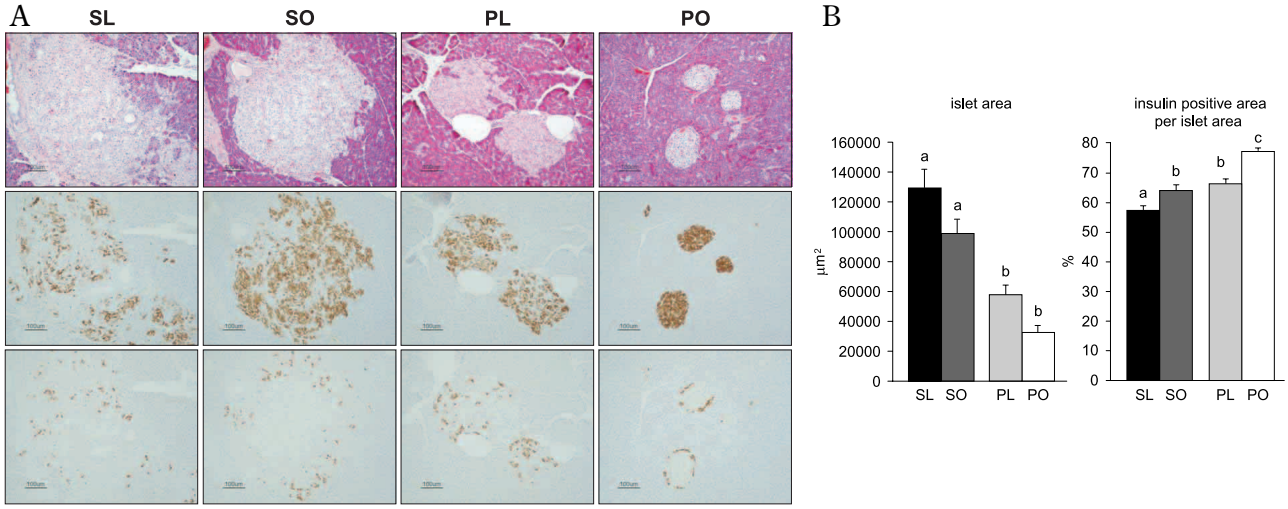

Fig. 3. Islet size, morphological β-cell mass and islet architecture in Zucker fatty rats fed combinations of carbohydrates and fats for four weeks
(A) Paraffin-embedded serial sections of pancreas from SL, SO, PL, or PO rats were stained with HE (upper row), or anti-insulin (middle row) or anti-glucagon antibodies (lower row). Hematoxylin counterstained. 4 μm-thick; magnification bar=100 μm. (B) Proportion of insulin-imunoreactive area to total islet area in pancreas. Twenty randomly chosen islets from each rat were analyzed. Values are means ± SEM, n=4 per group. Means with superscripts without a common letter differ, $P<0.001$.

portion of β-cell area to islet area in the PO group was significantly higher than others, whereas β-cell area was lowest in the SL group (Fig 3B).

Glucagon staining revealed abnormal islet-cell architecture in linoleic-acid-fed (PL and SL) rats; these rats had glucagon-positve α-cells distributed throughout islet that showed a disrupted mantle-like boundary structure. In contrast, the islet architecture of oleic-acid-fed (PO and SO) rats was nearly normal, with α-cells distributed only at the periphery of the islet. The peripheral mantle of α-cells was less dense and more centrally localized in SO rats compared to PO rats (Fig 3A).

Islet fibrosis

Using Masson's trichrome stain (blue staining), we assessed the degree of islet fibrosis (a common feature of several rat models of diabetes with progressive β-cell loss) to investigate the effects of dietary factors on the altered morphology and loss of β-cells (18, 19). Islet fibrosis was significantly more aggravated in sucrose-fed rats than in palatinose-fed rats (Fig. 4). Islets from rats fed the SL diet were disrupted at their boundaries and showed the greatest area of fibrosis, with some apparent in exocrine tissue as well (Fig. 4).

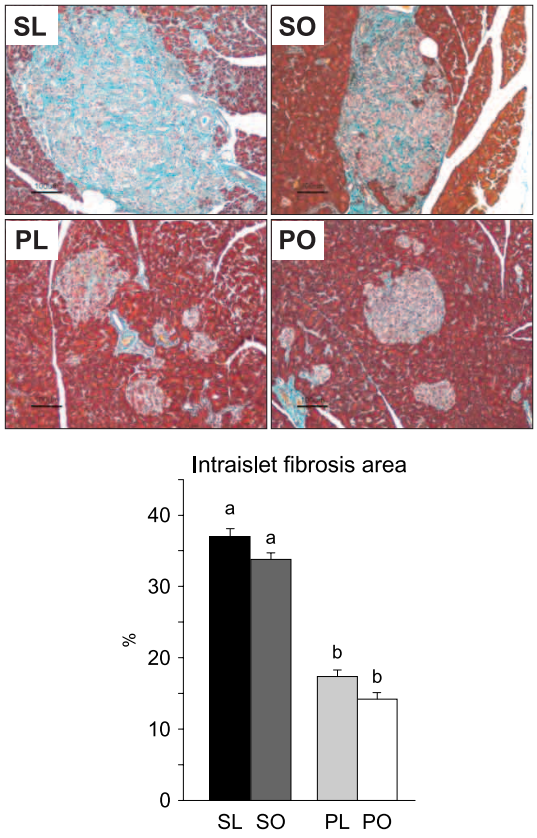

Fig. 4. Islet fibrosis in Zucker fatty rats fed combinations of carbohydrates and fats for four weeks
Masson's trichrome staining (collagen shown in blue) of paraffin-embedded pancreas sections from SL, SO, PL, or PO rats. 4 μm-thick, magnification bar=100 μm.

Islet cell apoptosis

Apoptosis has been shown to be the primary mechanism driving β-cell loss (20). Since pro-apoptotic caspase-3 activity is indicated by the nuclear localization of cleaved (activated) caspase-3 (20), we used immunohistochemical detection of activated caspase-3 in pancreatic islet to measure of β-cell apoptosis. In linoleic-acid-fed rats, intense immunostaining of nuclei around islet boundaries indicated increased β-cell apoptosis in these rats (Fig. 5). Relative to SL and PL rats, activated caspase-3 was detected sporadically in the islets of SO rats and infrequently in the islets of PO rats.

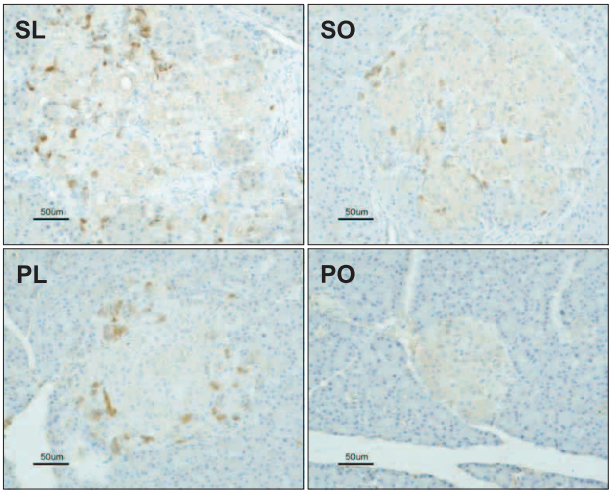

Fig. 5. Islet cell apoptosis in Zucker fatty rats fed combinations of carbohydrates and fats for four weeks
Paraffin-embedded pancreas sections from SL, SO, PL, or PO rats immunostained for pro-apoptotic activated caspase-3. Hematoxylin counterstained. 4 μm-thick, magnification bar=50 μm.

DISCUSSION

To assess the beneficial or adverse effects of specific dietary factors on metabolic disorders in obesity, we tested the effects of four diets that included one of two types of carbohydrates (palatinose or sucrose) and fats (oleic acid or linoleic acid) on Zucker fatty rats. Our findings demonstrate clearly that particular combinations of carbohydrate and fat vary with respect to their effects on metabolism and obesity.

After four weeks, all of the rats fed any of the four diets showed similar body and adipose tissue

weights, adipose cell size, and blood concentrations of glucose, insulin and lipids. This finding contrasts with our previous report, in which rats fed sucrose for eight weeks showed increased incidences of visceral fat accumulation, hyperglycemia, hyperinsulinemia and hyperlipidemia relative to those fed palatinose (12). We attribute the differences between 4-wk and 8-wk feeding results to time-dependent responses to carbohydrate loading. Previous reports are consistent with this hypothesis, in that feeding rats sucrose for a long term (15 wk or 20 wk) increased plasma glucose and insulin levels, body weight, and adipose tissue mass and cell size (21, 22), whereas sucrose feeding for a short term (3 wk or 4 wk) resulted in no significant changes.

Differences in dietary fatty acid profile have been reported to influence the fatty acid composition of adipose tissue and membrane phospholipids, as well as membrane protein function (23-26). Dietary linoleic acid has been reported to increase linoleic acid levels in rat adipose cell membrane phospholipids (23, 25, 26). Linoleic acid incorporated in phospholipids is known to be converted to arachidonic acid, an important precursor of eicosanoid lipid mediators (i.e., prostaglandins, hydroperoxyeicosatetraenoic acids (HPETEs), and leukotrienes) that play a pivotal regulatory role in inflammatory responses (27). In contrast, a diet rich in oleic acid has been reported to decrease arachidonic acid mobilization from membrane phospholipids and to reduce the production of prostaglandin E2 in rat macrophages (28). Similar to our results with carbohydrate feeding for only four weeks, dietary effects on inflammatory status that were evident after eight weeks of feeding rats a diet rich in linoleic acid (PL and SL) (but not oleic-acid) (12) also were not evident in this experiment. We hypothesize that changes in dietary fat that lead to membrane composition changes and modulate adipose tissue function may require a longer duration of feeding than the four-week period in this experimental procedure.

The pancreas was the only organ in which we observed striking morphological differences between rats in the four feeding groups : Pancreatic tissue sections from PO rats showed small oval islets with normal cell architecture ; PL pancreatic sections showed small but somewhat irregular islets with disrupted cell architecture and moderate β-cell loss ; SO rat islets were enlarged and irregular with almost intact cell architecture and moderate β-cell loss ; SL rat islets were hypertrophic and more irregular in shape, with abnormal cell architecture and progressive β-cell loss. These results were consistent with a previous study in which Zucker fatty rats that consumed SL and SO diets for eight weeks developed pancreatic islet hypertrophy, whereas those that consumed SL and PL showed significant reduction in β-cell area (12). These findings combined suggest that the pancreas is the initial site where dietary carbohydrates and fats act together to mediate dynamic changes in pancreatic islet formation via a mechanism that is independent of glycemic control or adiposity.

In Zucker fatty (*fa/fa*) rats, mutation of the leptin receptor gene results in animals that are hyperphasic, obese, hyperlipidemic, hyperinsulinemic and severely insulin-resistant, but not overtly hyperglycemic (29). These features make this strain a useful model for investigating islet β-cell compensation for insulin resistance. In a preliminary study, we confirmed that our experimental diets had no differential effects on pancreatic morphology in metabolically normal Sprague-Dawley rats by using the same procedure as used in the current study. Islet hyperplasia and hypertrophy are pathological changes in islet morphology that occur in response to obesity-associated insulin resistance. In both the current and previous studies using Zucker fatty rats, we observed that islets of sucrose-fed rats were significantly larger than those of palatinose-fed rats. In nutrient terms, the principal difference between sucrose and palatinose is their digestibility.

Palatinose is a naturally occurring α-1,6-linked glucose and fructose disaccharide that is isocaloric with sucrose (16.7kJ/g), but is absorbed more slowly. Although palatinose is completely hydrolyzed and absorbed, the hydrolysis rate of palatinose in rat and human intestine averages only 11-25 % (30), resulting in suppression of postprandial/fasted hyperglycemia and hyperinsulinemia relative to sucrose (4, 10, 11). Although the mechanistic details are unclear, the preventing effect of palatinose or aggravating effect of sucrose on islet hypertrophy may be due to the opposing effects of these sugars on glycemia and insulinotropy. In the current study, no differences in fasted glucose and insulin levels were noted between rats fed palatinose and sucrose for four weeks. Nevertheless, postprandial glucose and insulin levels, which were not measured, might have differed as reported

previously by our group (10, 11). Other investigators have also reported that sucrose and other high glycemic starch amylopectin can induce islet hypertrophy (31-33). Furthermore, amylopectin-fed rats had larger and more fibrotic islets than low glycemic-amylose-fed rats without decrease in insulin sensitivity (32).

Peripheral insulin sensitivity is a major determinant for islet morphology (34). Recently, hepatic fatty acid composition regulated by a long-chain fatty acid elongase, Elovl6, was reported as an independent determinant for obesity-induced insulin resistance (35). Although we did not measure hepatic fatty acid composition of the Zucker fatty rats that consumed our experimental diets, the hepatic expression levels of Elovl6 were similar among groups (data not shown). However, the careful assessments of pelipheral insulin sensitivity and tissue fatty acid composition will be needed to elucidate the dietary regulation of islet morphology in our experimental procedure.

Development of type 2 diabetes is associated with progressive histophathological changes in pancreatic islets, including selective loss of β-cells and fibrosis (36). The most obvious mechanism to explain pancreatic decompensation is a progressive loss of β-cell mass, which is hastened by islet fibrosis. Our data demonstrate that palatinose, relative to sucrose, had an antifibrotic effect on the islets of Zucker fatty rats : Sucrose-fed rats showed a much higher proportion of distinctly fibrotic islets than palatinose-fed rats, and SL islets exhibited more pronounced fibrosis than SO rats.

Our data agree with other studies demonstrating that feeding normal and diabetic rats sucrose and high-glycemic amylopectin led to islet fibrosis accompanied with islet hypertrophy (19, 32). In addition, *in vitro* studies showed that high concentration of glucose (which can result from sucrose feeding) induced pancreatic stellate cell (PSC) proliferation, resulting in increased extracellular matrix production and a consequent aggravation of fibrosis (37, 38). High glucose and high insulin have been reported to have an additive effect on the activation and proliferation of rat PSCs (39). The extensive fibrosis seen in SL rats may be due to a combination of dietary sucrose and linoleic acid inducing postprandial hyperglycemia and hyperinsulinemia simultaneously in response to linoleic acid potentiation of glucose-induced insulin secretion via the leukocyte type 12-lipoxygenase (12-LOX) pathway (40, 41).

Apoptosis is another major contributor to β-cell loss. Our structural studies clearly illustrated islets with pronounced apoptosis, as well as abnormal α-cell distribution, in linoleic-acid-fed rats relative to oleic-acid-fed rats. Because the specialized architecture and relative distribution of cell types within islets are likely to play important roles in islet function and survival, loss of cell-to-cell communication in disrupted islets may also promote apoptosis (42).

Glucolipotoxicity theory proposes that simultaneous increases in blood glucose and lipids result in an intracellular accumulations of lipid metabolites, which have highly deleterious effects on cell function and accelerate β-cell apoptosis (43). This theory links several dietary factors, including types of fat especially, closely to the development of β-cell apoptosis. β-cell apoptosis has been shown to be induced by saturated fatty acids (SFA ; palmitic acid, stearic acid) whereas unsaturated fatty acids (MUFAs, PUFAs) have no or little effects (9, 44-46). Eitel, *et al.* have reported that oleic and linoleic acids prevented SFA-induced β-cell apoptosis identically (44), and also that linoleic acid had no effect on caspase-3 activity (45). The basis for this inconsistency with our results may involve the 12-LOX pathway : Leukocyte type 12-LOX, which is expressed specifically in pancreatic β-cells, catalyzes the conversion of arachidonic acid and linoleic acid to the cytotoxic compounds 12-hydroperoxyeicostetraenic acid (12-HPETE) and 13-hydroperoxyoctadecadienoic acid (13-HPODE), respectively (47-49). Thus, dietary feeding with linoleic acid could promote an increase in the abundance of arachidonic-acid- and linoleic-acid-containing phospholipids in islets (26, 41), stimulating 12-LOX-induced formation of pro-apoptotic agents. Further studies will be needed to investigate the modulation of 12-LOX in pancreatic β-cells under these dietary conditions.

We hypothesize that an advancement of fibrosis coupled with an increase in apoptosis contribute to the striking β-cell loss seen in the islets of SL rats, and that an absence of both states may help preserve of β-cells in PO rats. Findings from obese Zucker rats fed for four versus eight weeks provide strong evidence that pancreatic islets may be target sites that translate the effects of different combinations of dietary carbohydrates and fats into metabolic changes. Our findings indicate that sucrose and linoleic acid together may act to induce subtle but striking changes in pancreatic is-

lets long before manifestation of type 2 diabetes symptoms. A combination of palatinose and oleic acid, which would help prevent deleterious changes in pancreatic islets, may provide a new dietary approach toward blocking links between obesity to type 2 diabetes.

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