Oral Nicotine Administration Decreases Tumor Necrosis Factor-Alpha Expression in Fat Tissues in Obese Rats

RunHua Liu, Takeshi Kurose, and Shigeru Matsukura

To investigate the effect of oral nicotine administration on insulin resistance and insulin secretion in an animal model of obesity, Zucker fatty rats were administered nicotine tartrate dihydrate orally through tap water (4.6 mg/kg/d, N group). Plasma nicotine concentrations in N group were 33.67 ± 10.49 ng/mL. The control (C) group consisted of pair-fed control rats. After 8 weeks of nicotine administration, both groups of rats were administered glucose (2 g/kg) orally in an anesthetized state, and blood was collected for glucose and plasma insulin measurements. The pancreases were isolated and perfused in vitro under pentobarbital anesthesia 1 week after glucose administration. The fat tissues were excised. The levels of tumor necrosis factor (TNF)-α protein were assessed using enzyme-linked immunosorbent assay (ELISA) or Western blot analysis. Serum leptin levels were measured using radioimmunoassay (RIA). Blood glucose levels in N group were significantly lower than in C group before and 120 minutes after glucose administration. The insulin secretion from the isolated perfused pancreases of N group appeared to be decreased compared with C group, but there was no significant difference. Histologic examination showed that the mean size of the pancreatic islets in N group was significantly smaller than in C group. The composition ratios of α and β cell mass of the pancreatic islets and fibroelastic tissues were not altered by nicotine administration. Portal TNF-α levels were comparable to peripheral levels in both groups. There were no significant differences in peripheral serum levels of TNF-α, free fatty acids (FFA), or leptin levels between N and C group. The TNF-α levels in visceral fat tissues in N group were significantly lower than those in C group. These results suggest that oral nicotine administration reduces insulin resistance in obese diabetic rats by decreasing production of TNF-α in the visceral fat tissues. Decreased islet size may be a secondary phenomenon induced by ameliorated insulin resistance, because the cellularity and fibroelastic tissues were not affected by the nicotine.

IT HAS BEEN SUGGESTED that physiologically active substances produced by adipose tissues, such as tumor necrosis factor-α (TNF-α), free fatty acids (FFA), and leptin, play a major role in the progression of insulin resistance in obesity.[1] TNF-α is one of the mediators of lipid metabolism,[2] adipocyte differentiation,[3] and in vivo insulin sensitivity.[4] TNF-α is expressed in macrophages and adipocytes and is substantially increased in obesity in both rodents[4] and humans.[5] Hotamisligil et al[4] have shown that in vivo neutralization of TNF-α ameliorates insulin resistance in Zucker fatty rats. In addition, administration of TNF-α to animals and humans results in reduced insulin sensitivity and causes insulin resistance.[6] Although TNF-α directly inhibits insulin signaling,[7] possibly through insulin receptor substrate-1 (IRS-1) phosphorylation,[8] the mechanism by which TNF-α induces insulin resistance remains unclear.

Fat tissues are divided roughly into 2 forms according to distribution, subcutaneous fat, and visceral fat. Of these, visceral fat is thought to be related to insulin resistance syndrome.[9] Although about 4,000 compounds occur in cigarette smoke,[10] nicotine is the most active alkaloid in tobacco, and it is well known that chronic cigarette smoking has direct effects on glucose metabolism,[11] especially on body weight.[12] The possible mechanisms of resistance to obesity induced by smoking[13] include reduced food intake,[14] increments in the amount of energy consumption due to metabolic change,[13] lipoprotein-lipase activation,[15] sympathetic nervous activation,[16] etc. Although these have been studied, the direct effect of nicotine on insulin sensitivity and insulin resistance has not been fully examined. In the present study, we have investigated the effects of long-term nicotine administration on insulin secretion in vivo and in vitro in a rat model of obesity. In addition, we examined the circulating levels of TNF-α, leptin, and FFA in blood, as well as the TNF-α levels in fat tissues as indicators of insulin resistance.

MATERIALS AND METHODS

Animals

Eight-week-old male Zucker fatty rats were housed in a humidity- and temperature-conditioned room with a 12 hour-light/dark cycle. The rats were divided into 2 groups of 10 animals.

Methods

The nicotine-administered group (N) received nicotine solution orally through tap water (nicotine tartrate dihydrate dissolved in distilled water, 4.6 mg nicotine/kg/d; the average intake of a rat in the N group was 2.4 mg nicotine/day) for 8 weeks. The control (C) group received water alone freely. Consumed food and water were measured every 2 days, and the body weight of both groups was measured at 9 AM to 11 AM every Monday morning. The 2 groups were pair-fed. The rats in the N group ate freely, and the average amount of rat chow consumed was given to each rat in C group. After 8 weeks of nicotine administration, the rats were starved overnight (20 hours). Both groups of rats were administered glucose (2 g/kg) orally through a tube inserted into the stomach. Blood samples for glucose and insulin measurements were obtained in an anesthetized state from the tail vein 0, 30, 60, and 120 minutes after oral glucose load. The rats were starved overnight and killed under pentobarbital anesthesia (intraperitoneal

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administration of 6 mg/100 g body weight of sodium pentobarbital) 1 week after the oral glucose tolerance test. The blood was collected from the peripheral and the portal vein, and fat tissues were excised in the fasting state. The pancreases were isolated and perfused with Krebs-Ringer bicarbonate buffer. The various fat tissues from the regions around the heart and the kidney and on the diaphragm, around the epididymis, mesenteries, and dorsal peritoneum were collected as visceral fat. Subcutaneous fat tissues from the interscapular, brachial, femoral, dorsal, and abdominal subcutaneous regions were also excised and stored immediately in liquid nitrogen (at −140°C).

Isolated Perfused Rat Pancreases

The pancreas was isolated as previously described.[17] The perfusate of Krebs-Ringer bicarbonate buffer containing 4.6% dextran (mean mol wt 70,000; Pharmacia, Uppsala, Sweden) was equilibrated with 95% O_2 and 5% CO_2, and the pH was adjusted to 7.4. It was placed in a reservoir maintained at 37°C. The glucose concentration was adjusted to the desired level by means of a side arm pump, and bovine serum albumin (fraction V; Sigma Chemical, St. Louis, MO) was added to the final concentration of 0.25%. The flow was nonrecycling, and the rate was generally adjusted at 1.9 mL/min. After excision, the perfused pancreas was placed in a temperature- and humidity-constant chamber. Each 1-minute effluent from the portal vein was collected into chilled tubes containing 1,000 U of Trasylol (Sigma), frozen immediately, and then stored at −30°C until assayed.

Insulin was measured by radioimmunoassay (RIA) using a polyethyleneglycol precipitation technique, with bovine insulin (Sigma) as standard.[18] Blood glucose was measured using the glucose oxidase method. The nicotine concentration in plasma was measured by gas chromatography with nitrogen selective detection.[19] Using a specific antibody (Antirat), serum TNF-α was measured by enzyme-linked immunosorbent assay (ELISA) with a kit obtained from R&D Systems (Minneapolis, MN), serum leptin was measured by RIA (Linco Research, St Louis, MO),[20] and serum FFA was measured by spectrophotometric assays using a commercially available kit obtained from Wako (Richmond, VA).

Western Blot Analysis

A total of 1 g of adipose tissue was homogenized in ice-cold buffer (50 mmol/L HEPES, 150 mmol/L sodium chloride, 1% Triton X-100 pH 7.8, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2.5 μg/mL pepstatin, and 5 μg/mL leupeptin, 10 μg/mL aprotinin), and the resulting homogenates were centrifuged for 20 minutes at 4°C at 12,000 rpm, aliquoted, and stored at −80°C until used. Samples containing 3.0 mg of protein were resolved by electrophoresis in a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Rat TNF-α (PEPRO TECH, Rocky Hill, NJ) and Rainbow markers (Amersham, Piscataway, NJ) were used as molecular markers. Proteins were transferred to polyvinylidene fluoride (PVDF; Millipore, Bedford, MA) membranes, and nonspecific binding sites were blocked by incubation in 5% (wt/vol) low fat dried milk dissolved in a buffer TBST (containing 50 mmol/L Tris-HCl [pH 7.6], 200 mmol/L NaCl, 0.2% Tween 20, and 0.1% SDS [TBST]) for 2.5 hours at room temperature. Filters were incubated with antimouse TNF-α polyclonal antibody (Endogen, Woburn, MA) at 0.65 μg/mL in low fat milk solution overnight at 4°C. After intensive washing in TBST, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated donkey antirabbit immunoglobulin G (IgG) (0.2 mg/mL [1/5,000], Amersham) in TBST-BSA. The membranes were then incubated with enhanced chemiluminescence (ECL, Amersham) reagents and exposed to x-ray film for 10 minutes. Densitometric analysis of immunoblots was performed using National Institutes of Health (NIH) Image 1.58 and Adobe PhotoShop (Adobe Systems) software for a Macintosh computer (Apple Computer, Inc). A TNF-α standard (0.1 μg) was run on every gel for comparison of samples from different immunoblots.

Histologic Study of Pancreatic Tissues

The pancreas was fixed by Bouin fixation liquid. After being embeded in paraffin, sections of the pancreas were cut 4 μm thick, and HE (hematoxylin-eosin) or Gomori and Azan staining was performed. Photographs of each of the 10 pancreatic islets were taken from every section at ×40 magnification, and the area of each pancreatic islet was calculated (long diameter × short diameter).

Statistical Analysis

Data are shown as mean ± SEM. Statistical analysis was performed by Student's unpaired *t* test for unpaired means obtained from 2 groups. Comparisons between means of a group were made using Student's paired *t* test. A *P* value less than .05 was accepted as statistically significant.

RESULTS

Body Weight and Food Intake

During 8 weeks of nicotine administration, there was no difference in food intake between the 2 groups (N group, 28.23 ± 2.37 g/day; C group, 29.86 ± 1.11 g/day). The body weight after 8 weeks of nicotine administration or water alone was the same in both groups (N group, 599.00 ± 59.48 g; C group, 627.50 ± 24.86 g) (Fig 1). The average amount of water consumed in each group was 24.26 ± 2.14 mL/day in N group and 25.63 ± 2.15 mL/day in C group.

Blood Glucose and Plasma Insulin

During the oral glucose tolerance test, blood glucose levels reached a peak value at 60 minutes in both groups after 2 g/kg oral glucose load. Blood glucose levels in N group were significantly lower than in C group before and 120 minutes after glucose administration (0 min, N group, 101.00 ± 2.41 mg/dL; C group, 131.80 ± 7.79 mg/dL, [$P < .01$]; 120 minutes, N group, 157.00 ± 17.47 mg/dL; C group, 219.40 ± 8.34 mg/dL,

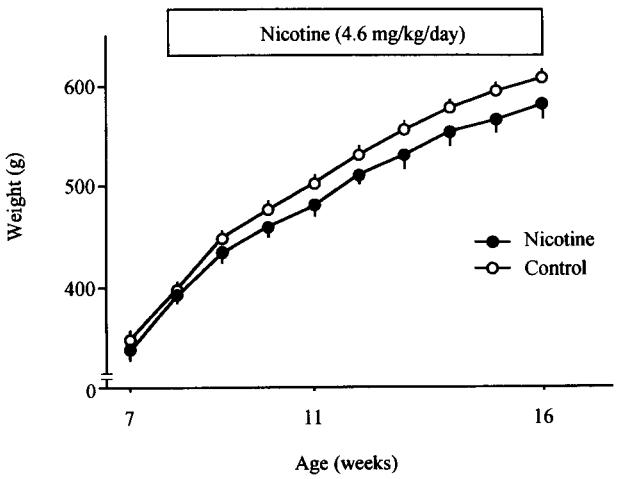

Fig 1. Effects of oral nicotine administration on body weight of Zucker fatty rats. Eight-week-old Zucker fatty rats were given nicotine tartrate dihydrate (4.6 mg/kg/d) in water from 8 to 16 weeks of age. Mean ± SE (n = 10). (●) Nicotine; (○) control.

[$P < .05$]). Plasma insulin levels reached a peak value of 8.04 ± 1.92 ng/mL at 30 minutes and fell thereafter in N group. Although plasma insulin levels in C group were lower than in N group, there was no significant difference at each time point (Fig 2). Both fasting blood glucose and the ratio of fasting insulin over glucose in N group were significantly lower than in C group (Table 1).

Isolated Perfused Rat Pancreas

The protocol and results are shown in Fig 3. When the glucose concentration in the perfusate was 5.5 mmol/L, the basal insulin secretion in C group was not significantly different from that in N group. The 16.7 mmol/L glucose-stimulated insulin secretion in both groups: the peak value in C group was 41.37 ± 7.86 ng/mL, slightly higher than that in N group (26.24 ± 5.59 ng/mL), although the difference was not significant statistically. In C group, the total increment above the basal level of insulin secretion during 16.7 mmol/L glucose infusion was similar to that observed in N group.

Plasma Nicotine Concentration

The plasma concentration of nicotine in the N group was 33.67 ± 10.49 ng/mL, whereas nicotine levels in C group were not detectable.

Serum Leptin, FFA, and TNF-α

Leptin levels in the portal vein were significantly lower than peripheral levels in both groups, however, there was no significant difference in leptin level between N and C group at the same collection sites. Both peripheral and portal levels of FFA in N group were comparable to those in C group, respectively. There was also no significant difference in peripheral or portal levels of TNF-α between N and C group (Table 2).

Histologic Examination of Pancreatic Islets

The mean area of each 10 pancreatic islets from N group (51.95 ± 8.20 μm^2) was significantly less than that from C group (112.93 ± 13.48 μm^2, $P < .01$). The percentages of α and β cell area in a pancreatic islet (about 15% and 70%, respectively) and fibroelastic tissues (about 15%) were similar in both groups (Fig 4A and B).

Table 1. Fasting Blood Glucose, Fasting Plasma Insulin and the Ratio of Fasting Insulin/Glucose in N and C Groups

Group	Fasting Blood Glucose (mg/dL)	Fasting Plasma Insulin (ng/mL)	Fasting Insulin/ Glucose ($\times 10^{-6}$)
Nicotine	87.30 ± 3.16*	8.61 ± 0.67	9.90 ± 0.77*
Control	99.50 ± 4.79	14.49 ± 2.73	13.90 ± 1.70

NOTE. Data are the mean ± SE for 10 rats.
*$P < .05$ *v* control.

Expression of TNF-α Protein

In the fat tissues of C group, TNF-α protein was clearly expressed in both intraabdominal and subcutaneous fat tissues. The TNF-α protein level in pericardial fat tissues was remarkably increased compared with the other subcutaneous or intraabdominal fat tissues. The TNF-α protein level in interscapular fat also was increased compared with the other subcutaneous fat tissues of C group. However, the TNF-α protein levels in the other subcutaneous and intraabdominal fat tissues were similar (Fig 5A and B).

The TNF-α protein level in abdominal subcutaneous fat in the N group was significantly lower than C group. However, the TNF-α protein levels in other subcutaneous fat tissues from N group did not differ from those of C group (Fig 6A and B). On the other hand, the TNF-α protein levels in all visceral fat tissues (renal, mesenteric, epididymal, heart, dorsal peritoneal, and diaphragmatic) in N group were significantly lower than in C group (Fig 6C and D).

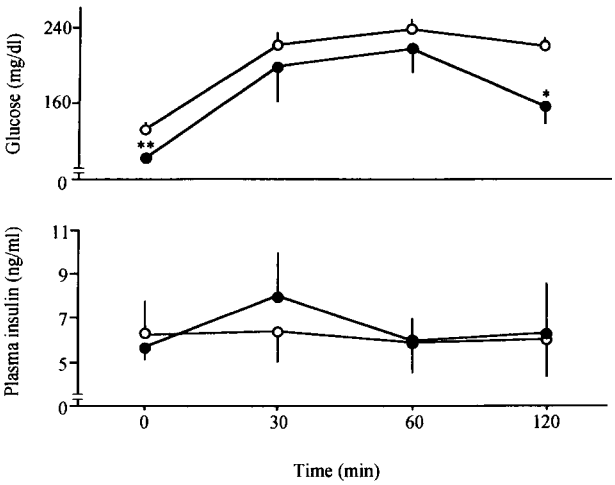

Fig 2. Plasma glucose and insulin levels in oral glucose tolerance test in N (●) and C (○) groups. Mean ± SE (n = 5) are shown. *$P < .05$, **$P < .01$ *v* control rats.

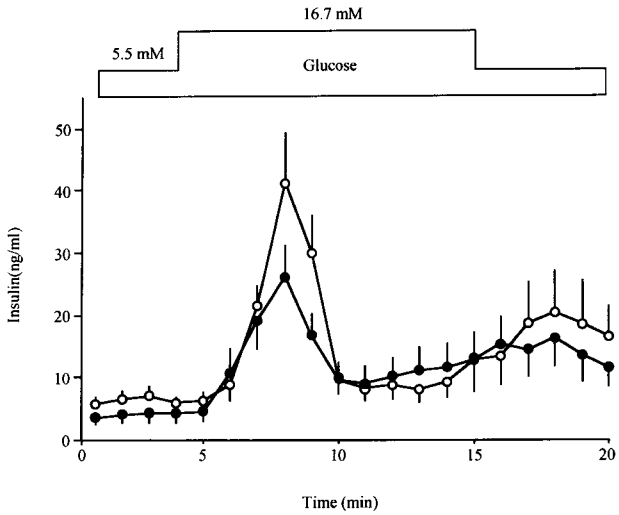

Fig 3. Glucose-stimulated insulin secretion from the isolated perfused rat pancreas in N (●) and C (○) groups. Mean ± SE (N, n = 7; C, n = 6).

Table 2. Peripheral and Portal Levels of TNF-α, Leptin, and FFA in N and C Groups

Group	TNF-α (pg/mL)		Leptin (ng/mL)		FFA (mEq/L)	
	Peripheral	Portal	Peripheral	Portal	Peripheral	Portal
Nicotine	74.31 ± 9.76	76.23 ± 12.78	75.89 ± 5.93	61.29 ± 5.35*	0.95 ± 0.08	0.95 ± 0.06
Control	93.23 ± 13.87	92.27 ± 13.32	87.66 ± 4.40	72.38 ± 3.87*	1.08 ± 0.15	0.92 ± 0.11

NOTE. Data are the mean ± SE for 10 rats.
*$P < .05$ *v* peripheral levels.

DISCUSSION

In the present study, oral glucose load in obese rats after long-term oral administration of nicotine elicited a decreased response of blood glucose in vivo. Plasma insulin levels, however, were not significantly different at each time point after oral glucose load in the obese rats. Furthermore, nicotine did not affect the total output of glucose-stimulated insulin secretion from the isolated perfused pancreas in vitro.

There have been several studies on the effect of nicotine and smoking on insulin secretion. Swislocki et al[21] reported that nicotine implanted subcutaneously did not alter glucose tolerance or insulin action in adult male Sprague-Dawley rats in vivo. Epifano et al[22] reported that the insulin secretion stimulated by glucagon was not influenced by smoking or subcutaneous injection of nicotine in type 2 diabetic patients. However, some contradictory results have been reported that nicotine tends to decrease the basal and stimulated insulin secretion.[23,24] However, these discrepancies may be explained by a variety of factors, including the degree of insulin resistance, species differences, autonomic neural state, and the amount of nicotine administered.

It has been reported that the high concentration of nicotine inhibited insulin secretion induced by glucose, while the low concentration of nicotine stimulated insulin secretion in rabbit pancreas.[25] We selected a low dose of nicotine, orally administered for 8 weeks, in this study because of previous studies.[23] The plasma nicotine levels in our experiments are comparable to those obtained from smokers in the human study.[26] In addition, the nicotine dose was equivalent to that delivered by 2 cigarettes, and it produced the venous plasma nicotine concentrations in the 10 to 40 ng/mL range observed in regular smokers.[27]

On the other hand, there are many studies on the effects of nicotine and smoking on insulin resistance. Smoking has been shown to impair insulin action and lead to insulin resistance.[26,28] Although nicotine is a main ingredient in cigarette smoke, nicotine itself seems to have contradictory effects on the induction of insulin resistance. Attvall et al[26] reported that plasma nicotine levels are similar in smoking and snuffing (portion-bag packed snuff, average nicotine content, 1 mg). Smoking, but not snuffing, impaired insulin action mainly due to a lower peripheral glucose uptake.[26] Furthermore, Swislocki et al[21] reported that smokeless nicotine is not associated with the deterioration in glucose tolerance in these rats. However, long-term use of nicotine gum has been reported to induce hyperinsulinemia and insulin resistance in man.[28]

However, in the present study, oral nicotine administration was found to significantly decrease the ratio of fasting insulin over glucose level, suggesting that nicotine mitigates the insulin resistance in the rats. Accordingly, we further examined the serum FFA, leptin, and TNF-α levels in the blood and found that these parameters of insulin resistance were not significantly different in the 2 groups. We then examined the TNF-α levels in the fat tissues. Among many special features, visceral fat is characterized by rapid turn-over and high sensitivity to catecholamine.[29] An increased visceral fat mass has been shown to have a close correlation with the development of insulin resistance syndrome.[29] Accordingly, the high expression of TNF-α protein in visceral fat could be the primary cause of the development of insulin resistance in this rat model of obesity. TNF-α is known to be one of the major mediators of insulin resistance.[8,30] Although the mechanism underlying the development of TNF-α–induced insulin resistance is not com-

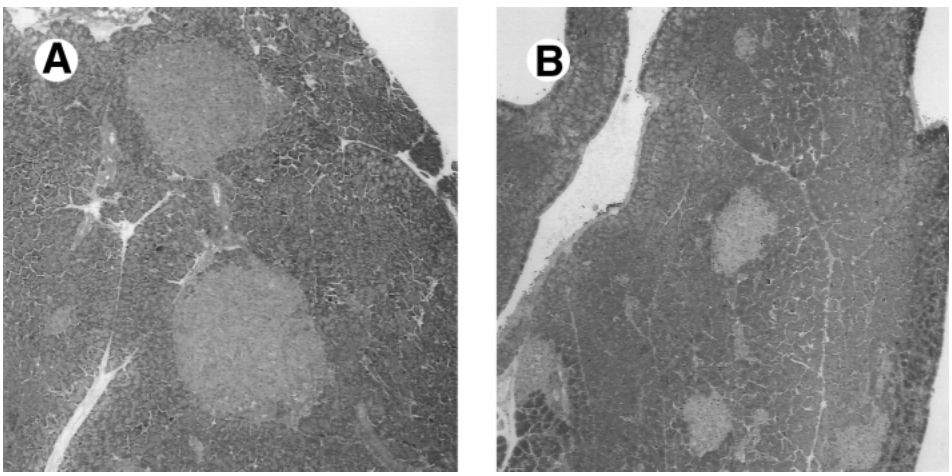

Fig 4. HE stain of pancreatic islets in (A) the C group and (B) the N group. Original magnifications ×40.

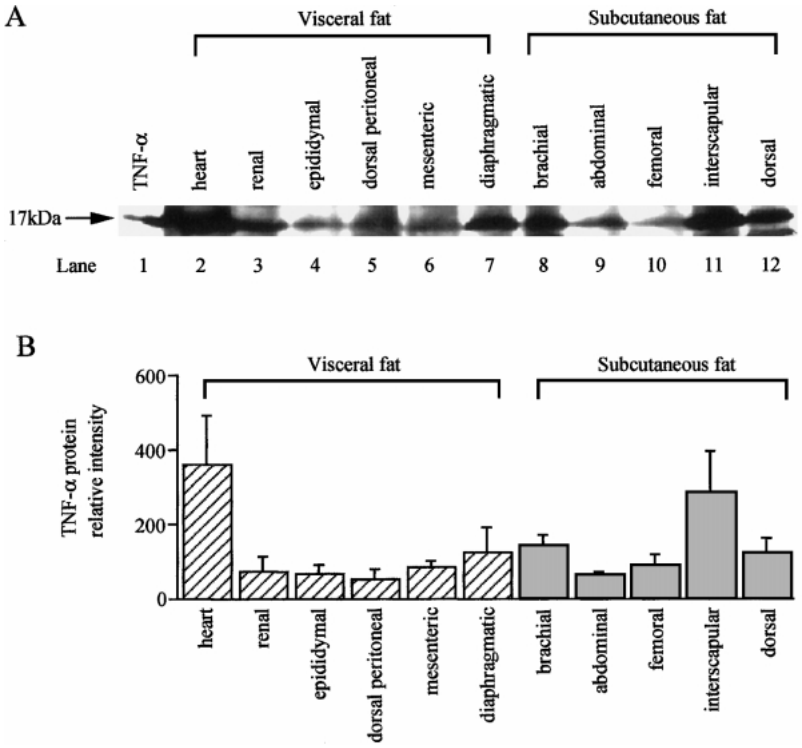

Fig 5. (A) TNF-α levels in the adipose tissues detected by Western blotting in C group. A total of 0.1 μg of TNF-α was applied as a molecular marker in Lane 1. Lanes 2 to 12, sample of the fat tissues. (B) Relative signal intensities of TNF-α protein in the Western blot. The signal intensity of the band was quantified using NIH image and Adobe PhotoShop software, and the percentage ratio to the corresponding standard TNF-α was calculated as a TNF-α protein relative intensity. Densitometric values are means ± SE of data obtained from 5 individual membrane preparations each performed with adipose tissues from 5 rats in each experimental group.

pletely clear, TNF-α is thought to act through paracrine rather than endocrine mechanisms. In obese animal models, TNF-α protein is elevated locally, as well as systemically, although the circulating levels are low (generally < 200 pg/mL).[4] While obese db/db mice have higher average circulating TNF-α levels than control mice, the concentration of plasma TNF-α (20 to 200 pg/mL) is well below that required to suppress insulin action in cultured cell systems.[7] The serum TNF-α levels in our study were also well below the level suppressive of insulin action, and they were not influenced by nicotine administration. Recently, it has been reported that the plasma concentration of soluble TNF receptors also increased in the insulin-resistant state.[31,32] Although in our present study, plasma levels of TNF-α were not influenced by nicotine, the soluble receptors may change with nicotine treatment, because work in humans has shown differences in TNF soluble receptor levels even when circulating TNF-α levels remain unchanged.[32]

The amount of TNF-α protein in the visceral fat tissues was significantly reduced by nicotine administration in the present study, the mechanism of which remains to be clarified. Sueoka et al[33] reported that new category compounds containing sugar-ester in tobacco leaf extracts inhibit the expression of the TNF-α protein. Many studies indicate that production of cytokines, including TNF-α from mononuclear cells, are decreased by nicotine, and that the promotion of tumor and inflammation are inhibited by nicotine.[34,35] The direct mechanism of insulin resistance induced by TNF-α and its mitigation by nicotine in these rats remains to be clarified.

The peripheral and portal levels of FFA and leptin were not altered in nicotine-administered rats in the present study. The effects of nicotine on the peripheral level of FFA and leptin, however, have been reported inconsistently. Oeser et al[36] found that adult smokers consuming an isocaloric diet after nicotine abstinence for 7 days showed no change in body weight or circulating concentrations of leptin or FFA. It has been reported that the circulating concentration of leptin showed a positive correlation with body mass index (BMI) and body adiposity.[37] Eliasson et al[38] found that long-term use of nicotine was associated with elevated circulating leptin levels in man. On the other hand, Wei et al[39] have reported lower circulating leptin concentrations in smokers than nonsmokers. In these cross-sectional studies, the lower levels of leptin persisted after correction of the lower BMI values in the smokers. It is possible that the smokers in these studies had less body fat than the nonsmokers at a given BMI, and that the lower leptin levels in these smokers is a reflection of their lesser adiposity. In cigarette smokers, leptin production from intraabdominal fat is reduced compared with that from the subcutaneous adipose tissue.[40] Hence, it is possible that the lower plasma leptin concentrations found in smokers may be related to the decreased mass of intraabdominal adipose tissue. In our present study, oral nicotine administration itself did not directly affect circulating leptin levels in Zucker fatty rats. Accordingly, our findings suggest that the total adiposity and intraabdominal fat mass of both groups are similar, and that leptin did not participate in the mechanism of the increased insulin sensitivity.

The size of the pancreatic islets is significantly reduced in nicotine-administered rats. Because it is well known that islet size is regulated by the peripheral insulin sensitivity,[41] this reduction in size should be a result of the increased insulin sensitivity rather than a direct effect of nicotine. Because the cellularity and fibroelastic tissues were not affected by nicotine

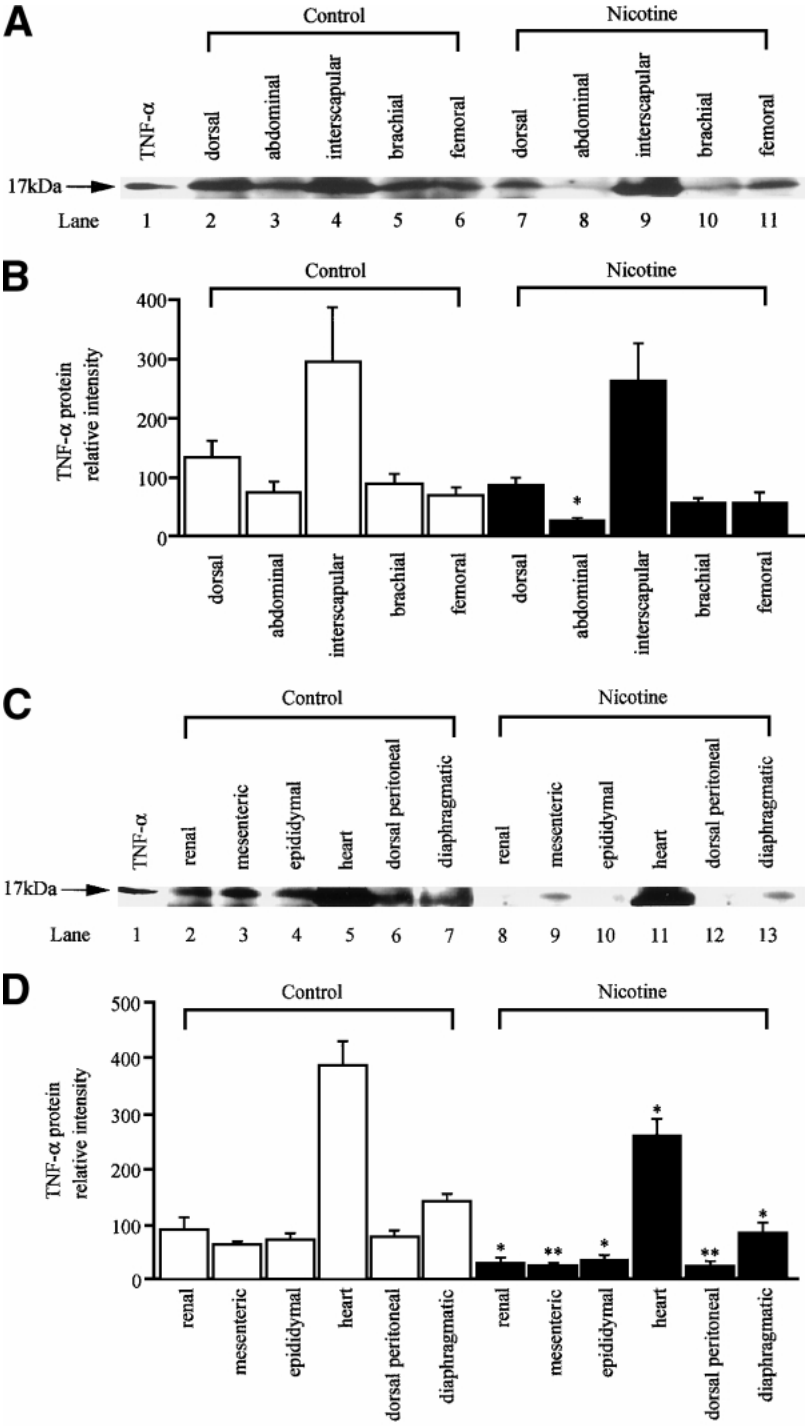

Fig 6. Western blotting for TNF-α in the subcutaneous (A) and visceral (C) adipose tissues from C or N group. A total of 0.1 μg of TNF-α was applied as a molecular marker in Lane 1. Lanes 2 to 11 (A) and Lanes 2 to 13 (C) sample of the fat tissues. (B, D) Relative signal intensities of TNF-α protein in Western blot are shown in C (white bars) or N (black bars) group. The signal intensity of the band was quantified using NIH image and Adobe PhotoShop software, and the percentage ratio to the corresponding standard TNF-α was calculated as a TNF-α protein relative intensity. Mean ± SE for 5 rats. *P < .05, **P < .01 *v* the same adipose tissue in C group.

administration, the reduced insulin resistance may lead to an increase in insulin sensitivity, in turn, reducing insulin secretory reserve.

In conclusion, our results indicate that oral nicotine administration decreases plasma glucose during fasting and after glucose load. Oral nicotine administration reduces insulin resistance in obese diabetic rats probably through decreased production of TNF-α protein, especially in the visceral fat tissues. The mechanism of insulin resistance by TNF-α and its mitigation by nicotine in these rats remains to be clarified.

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