

Long-term oral nicotine administration reduces insulin resistance in obese rats

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Received 15 July 2002; received in revised form 4 November 2002; accepted 6 November 2002

Abstract

This study aimed to investigate the effect of long-term oral nicotine administration on insulin resistance in an animal model of obesity. Eight-week-old male Zucker fatty rats (ZFRs) were administered nicotine tartrate dihydrate (4.6 mg/kg/day) in the drinking water. The control group was pair-fed. The body weights and food intake over 8 weeks were similar in both groups. Plasma glucose levels at 3, 6, 9, 12, and 15 min after insulin administration (0.5 U/kg) in the nicotine group were significantly lower than those in the control group. The calculated K_{ITT} value for the nicotine group was significantly higher than that for the control group. Wet weight of the liver in the nicotine group was significantly lower than that in the control group. Transaminases and histological examination of the liver revealed no alteration by nicotine administration. Glycogen, glycogen synthetase activity and gluconeogenesis in the liver in the nicotine group were significantly lower than those in the control group. Phosphorylase-a activity of the liver in the nicotine group was significantly higher than that in the control group. Glycogen, glycogen synthetase, and phosphorylase-a activity of skeletal muscle were similar in both groups. These results suggest that long-term oral nicotine administration may reduce insulin resistance in obese diabetic rats through a reduced hepatic glucose release and, in part, contribute to lowering blood glucose levels.

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Keywords: Nicotine; Insulin resistance; Obesity; Glycogen; Zucker fatty rat

1. Introduction

The Zucker fatty (fa/fa) rat (ZFR) develops obesity and diabetes because of a failure to respond to leptin (Halaas et al., 1995) due to a mutation in the receptor gene that is expressed in the hypothalamus (Phillips et al., 1996), although *ob* gene expression and leptin secretion are markedly augmented (leptin resistance) (Couillard et al., 2000). ZFR is considered to be a good model for type 2 diabetes mellitus in the early stage because it shows both hepatic and peripheral insulin resistance (Terrettaz et al., 1986). The insensitivity to insulin and relative insulin deficiency in type 2 diabetes lead to a decrease in glucose utilization by liver, muscle, and adipose tissue and to an increase in hepatic glucose production (McGarry, 1992). It is well known that skeletal muscle is a major tissue responsible for insulin-mediated glucose uptake (Shulman et al., 1990) and that defects in insulin-stimulated muscle glycogen synthesis are

largely responsible for the insulin resistance observed in obese patients with type 2 diabetes (Paquot et al., 2000). In liver, glycogen is mainly stored as a reserve of glucose for extrahepatic tissues during starvation (Bollen et al., 1998). Excessive hepatic glucose output is an important factor in the fasting hyperglycemia of type 2 diabetes (DeFronzo, 1988). The liver glycogen content in ZFR is controversial. It has been reported that the liver glycogen content is higher in the ZFR than in lean rats (Triscari et al., 1980). On the other hand, there is a report that liver glycogen content is lower in the ZFR than in lean rats (Aiston et al., 2000). A recent study showed that leptin has additive effects with insulin in inhibiting phosphorylase and stimulating glycogen storage in hepatocytes, indicating that the primary action of leptin in hepatocytes is to enhance glycogen storage (Aiston and Agius, 1999). Chen et al. (1993) reported that increased glycogen storage in the liver contributes to the development of insulin resistance and glucose intolerance in obese mice. Several studies suggested that the contribution of gluconeogenesis to hepatic glucose output is increased, and plays a major role in the augmented plasma glucose concentration during the postabsorptive state in type 2 diabetes (Consoli et

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al., 1987, 1989) and diabetic animals (Wyse and Dulin, 1974).

Although approximately 4000 compounds occur in cigarette smoke, nicotine is the most active alkaloid in tobacco, and it is well known that chronic cigarette smoking has direct effects on glucose metabolism (Jensen et al., 1995) and on body weight (Carney and Goldberg, 1984). The possible mechanisms of resistance to obesity induced by smoking (Hofstetter et al., 1986) include reduced food intake (Wager-Srdar et al., 1984), increases in the amount of energy consumption due to metabolic changes (Hofstetter et al., 1986), lipoprotein-lipase activation (Brunzell et al., 1980), and sympathetic nervous activation (Wack and Rodin, 1982). Although these aspects have been studied, the direct effect of nicotine on insulin resistance has not been fully examined. In the present study, we investigated the effects of long-term nicotine administration on insulin resistance in liver and muscle in a rat model of obesity. We measured various parameters of glucose metabolism and key regulatory enzymes involved in glycogen synthesis, such as glycogen synthetase and phosphorylase-a activity in liver and muscle as well as hepatic gluconeogenesis in obese rats after long-term oral nicotine administration.

2. Materials and methods

2.1. Animals

Eight-week-old male ZFRs (Experimental Animal laboratory, Tokyo, Japan) were housed in a humidity- and temperature-conditioned room with a 12-h light/dark cycle. The rats were divided into two groups of 11 animals each.

2.2. Materials

UDP-[U-¹⁴C]-glucose and glucose-1-phosphate ¹⁴C were purchased from DuPont NEN (Life Science Products, Boston, MA). Glucose-6-phosphate, NaF and anthrone were from Wako (Japan). Rabbit liver glycogen and oyster liver glycogen were purchased from Sigma (Linco Research, St. Louis, MO, USA). NaH¹⁴CO₃ was purchased from New England Nuclear (Boston, MA). All other reagents were of the highest purity available.

2.3. Methods

The nicotine-treated group received nicotine solution orally in tap water (nicotine tartrate dihydrate dissolved in water to a concentration of 4.6 mg nicotine/kg/day so that the average intake of a rat in the nicotine group was 2.4 mg nicotine/day) for 8 weeks. The control group received water ad libitum. Consumed food and water were measured every 2 days, and the body weight of both groups was measured at 9:00–11:00 AM every Monday morning. The two groups were pair-fed. The rats in the nicotine group ate freely, and

the average amount of chow consumed in this group was given to each rat in control group. After 8 weeks of nicotine administration, the rats were starved overnight. Four rats from each group were anesthetized (i.p. 6 mg/100 g body weight of sodium pentobarbital) and injected with NaH¹⁴CO₃ (20 μCi/100 g body weight) into the femoral vein. Blood samples (0.5 ml) were obtained from another femoral vein at 10, 20, 30, 45, and 60 min after NaH¹⁴CO₃ administration. Another four rats from each group were anesthetized and injected with insulin (0.5 U/kg, Humulin R, Eli Lilly, Kobe, Japan) into the femoral vein. Blood samples for glucose measurements were obtained from the thigh vein –10, 0, 3, 6, 9, 12, 15, 30, and 60 min after insulin injection. The rats were starved overnight and killed under pentobarbital anesthesia 1 week after the insulin tolerance test. Peripheral blood was collected for biochemical analyses, and skeletal muscle (soleus) and organs (pancreas, kidney, heart, and liver) were excised and stored immediately in liquid nitrogen. Glycogen, glycogen synthetase and phosphorylase-a activity of liver and muscle were assayed. Hematoxylin–eosin staining and glycogen staining were performed on histological sections of the liver.

Plasma glucose was measured using the glucose oxidase method. Insulin sensitivity was assessed from the *K* index of the insulin tolerance test (*K*_{ITT}). The rate constant for plasma glucose disappearance (*K*_{ITT}) was calculated from the formula $K_{ITT} = 0.693/t_{1/2}$ (Matsumoto et al., 1999). Plasma glucose *t*_{1/2} was calculated from the slope by least square analysis of the plasma glucose concentrations from 3 to 15 min after i.v. insulin injection, when the plasma glucose concentration declined linearly. The plasma nicotine concentration was measured by gas chromatography with nitrogen-selective detection (Curvall et al., 1982). Serum transaminase, γ-glutamyl transpeptidase, lactic dehydrogenase, alkaline phosphatase, triglyceride, and total cholesterol were measured using an enzymological method. Serum total protein was measured with the biuret method. Serum albumin was measured using the bromocresol green method. Serum cholinesterase was measured by ultraviolet (UV) absorption. Serum free fatty acid was measured with an enzymological assay (acyl-CoA synthetase-acyl-CoA oxidase) using a commercially available kit from EIKEN KIZAI (Japan).

2.4. Histological study of liver tissues

The liver was fixed with formalin phosphate-buffered saline fixation liquid. After embedding in paraffin, 3–4-μm-thick sections were cut for hematoxylin–eosin or periodic acid Schiff glycogen staining. Photographs were taken at × 40 magnification.

2.5. Glycogen assay

Liver tissue, 1 g, was added to 3 ml of 30% KOH solution. The tissue was then heated in boiling water for 20

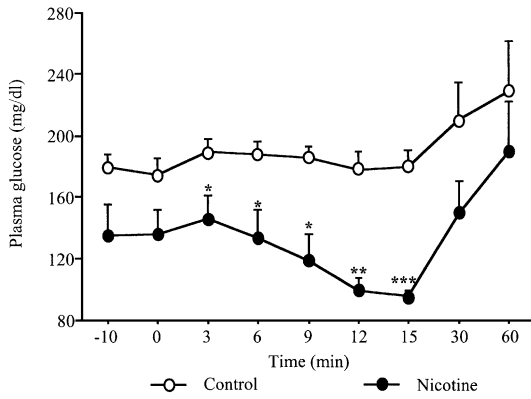


Fig. 1. Plasma glucose levels in insulin tolerance test in nicotine and control groups. Means \pm S.E.M. ($n=4$) are shown. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. control group.

to 30 min. When the tissues were dissolved, 0.5 ml of saturated sodium sulfate was added and glycogen was precipitated by the addition of 4 ml of 95% ethanol. The tube and contents were heated again until the mixture began to boil, then cooled and centrifuged at 3000 rpm, 4 °C. The precipitated glycogen was redissolved in 2 ml of distilled water, reprecipitated with 2.5 ml of 95% ethanol and centrifuged. The alcoholic supernatant was decanted off and the precipitate was stored at -80 °C until determination of glycogen with anthrone reagent was performed (Hassid and Abraham, 1957).

2.6. Glycogen synthetase assay

Liver tissue, 0.5 g, was homogenized in 6 ml of ice-cold buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 100 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and the resulting homogenates were centrifuged for 20 min at 4 °C at 12000 rpm, and stored at -80 °C until used.

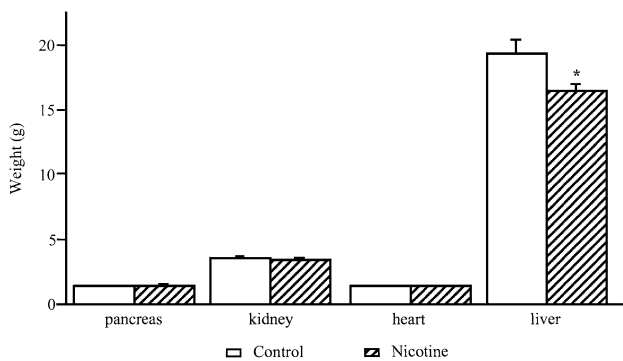


Fig. 2. Effects of oral nicotine administration on the wet weight of visceral organs from Zucker fatty rats. Eight-week-old Zucker fatty rats were given nicotine tartrate dihydrate (4.6 mg/kg/day) in water from 8 to 16 weeks of age. Means \pm S.E.M. ($n=11$). * $P<0.01$ vs. control group.

Table 1
Biochemical values for peripheral blood serum in control and nicotine groups

	Total protein (g/dl)	A/G	Bilirubin (mg/dl)	GOT (IU/l)	GPT (IU/l)	LDH (IU/l)	ALP (IU/l)	γ -GTP (IU/l)	Cholinesterase (IU/l)	Triglyceride (mg/dl)	Cholesterol (mg/dl)	FFA (mEQ/l)
Control	6.30 \pm 0.24	1.85 \pm 0.29	0.32 \pm 0.08	135.00 \pm 17.09	54.00 \pm 2.31	2392.83 \pm 616.81	201.67 \pm 32.17	3.67 \pm 1.47	5.00 \pm 0.57	215.25 \pm 73.41	115.5 \pm 7.19	0.88 \pm 0.17
Nicotine	6.67 \pm 0.15	2.47 \pm 0.09	0.33 \pm 0.12	164.67 \pm 12.91	55.00 \pm 7.23	3287.67 \pm 460.00	178.33 \pm 16.13	3.71 \pm 0.84	5.33 \pm 0.33	145.50 \pm 20.42	99.25 \pm 6.82	0.76 \pm 0.06

Data are presented as means \pm S.E.M. ($n=7$).

A 25- μ l aliquot of the tissue extract was mixed with 25 μ l of buffer [67 mM Tris–HCl, pH 7.8, 6.7 mM EDTA, 13 mg/ml oyster glycogen, 8.9 mM UDP-[U- 14 C]-glycogen (1 μ Ci/ μ mol), and with/without 10 mM glucose-6-phosphate]. The mixture was incubated for 30 min at 30 °C. The reaction was terminated by the addition of 60% KOH and the mixture was then boiled for 20 min. Glycogen was precipitated with ethanol containing 1% (w/v) NaCl, and the pellet was dissolved in 1 ml of H₂O, and radioactivity was counted using a scintillation counter. The total activity of glycogen synthetase was measured in the presence of glucose-6-phosphate, an allosteric activator of the enzyme. The activity of the dephosphorylated form of glycogen synthetase was measured without the addition of glucose-6-phosphate. The glycogen synthetase ratio: activity without the addition of glucose-6-phosphate / activity with the addition of glucose-6-phosphate/activity without the addition of glucose-6-phosphate, was used to determine the activation state of glycogen synthetase in vivo (Thomas et al., 1968).

2.7. Glycogen phosphorylase-a assay

The tissues were homogenized first with 2 vol. homogenizing solution I containing 50 mM potassium fluoride and 10 mM EDTA in 60% glycerol, pH 7.0, at 0 °C. Then 10 vol. homogenizing solution II (50 mM potassium fluoride and 10 mM EDTA in water, pH 7.0) was added and homogenization was completed at 4 °C. The homogenate was centrifuged at 12000 rpm for 10 min (4 °C). The supernatant was either frozen at –80 °C until used or used immediately for assay. To assay for total phosphorylase-a

activity, 30 μ l of the diluted enzyme preparation (1:40) was added to 60 μ l of an assay mixture containing 200 mM potassium fluoride, 100 mM glucose 1-phosphosphate- 14 C (0.0025 μ Ci/ μ mol), 1% rabbit liver glycogen. Glycogen phosphorylase-a activity was assayed by the method of Gilboe et al. (1972).

2.8. Determination of gluconeogenic activity

Gluconeogenic activity was measured as described by Fujiwara et al. (1995). Blood samples (0.5 ml) were hemolyzed in 6 ml distilled water and then deproteinized by the addition of 1 ml 5% Ba(OH)₂ and 1 ml 5% ZnSO₄ before centrifugation at 3000 rpm for 5 min. Then, 2 ml of this supernatant was removed and placed into tubes containing anion-exchange resin (AG-8X, formate form, 200–400 mech; Bio-Rad, Hercules, CA, USA). The tube was shaken occasionally for 20 min, then the supernatant was transferred into another tube containing cation-exchange resin (AG50W-8X, H⁺ form, 200–400 mech; Bio-Rad). Radioactivity (14 C-glucose) in the final supernatant was measured in a liquid scintillation counter (LSC-5100; ALOKA, Tokyo, Japan) using aqueous counting scintillation (ACSII) solvent (Amersham, IL, USA).

2.9. Statistical analysis

Data are shown as means \pm S.E.M. Statistical analysis was performed with Student's unpaired *t*-test for unpaired means from two groups. Comparisons between means within a group were made using Student's paired *t*-test. A *P* value less than 0.05 was accepted as statistically significant.

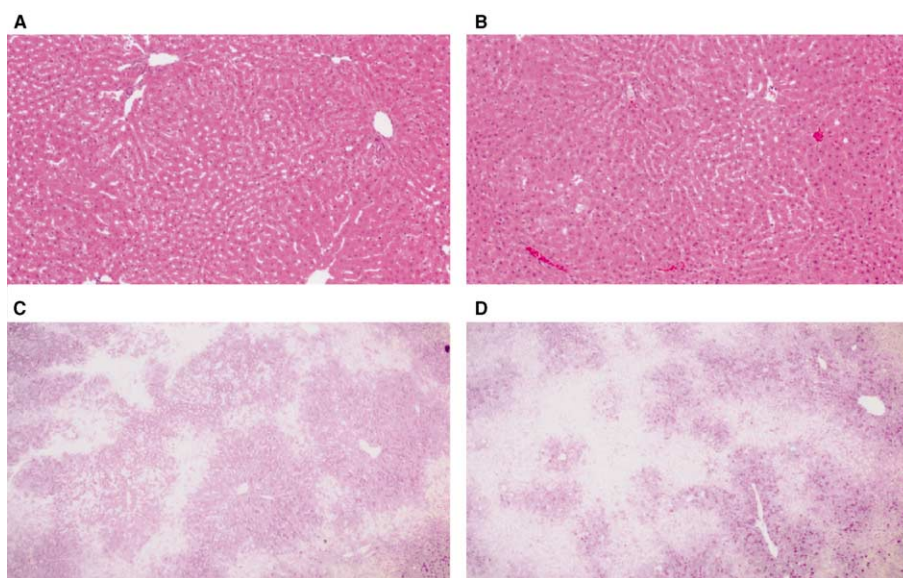


Fig. 3. (A) Hematoxylin–eosin stain of liver in control group ($\times 40$). (B) Hematoxylin–eosin stain of liver in nicotine group ($\times 40$). (C) Periodic acid Schiff stain of liver glycogen in control group ($\times 40$). (D) Periodic acid Schiff stain of liver glycogen in nicotine group ($\times 40$).

3. Results

3.1. Body weight and food intake

During 8 weeks of nicotine administration there was no difference in food intake between the nicotine and control groups (Nicotine group: 28.23 ± 2.37 g/day, Control group: 29.86 ± 1.11 g/day). There was also no difference in body weight between the two groups (Nicotine group: 550.91 ± 13.38 g, Control group: 573.18 ± 13.19 g). The average amount of water consumed in each group was 24.26 ± 2.14 ml/day in the nicotine group and 25.63 ± 2.15 ml/day in the control group.

3.2. Insulin tolerance test

Basal plasma glucose levels showed no difference between the nicotine and control group. Plasma glucose levels at 3, 6, 9, 12, and 15 min after insulin administration in the nicotine group were significantly lower than those in the control group (Fig. 1). The calculated K_{ITT} value for the nicotine group was significantly higher than that for the control group ($3.60 \pm 0.69\%$ vs. $0.70 \pm 0.49\%$ min^{-1} , $P < 0.05$).

3.3. Plasma nicotine concentration

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

3.4. Wet weight of organs

Wet weights of the pancreas, kidney, and heart were similar in both groups. The weight of the liver in the nicotine group was, however, significantly lower than that of the liver in the control group (16.41 ± 0.47 vs. 19.34 ± 0.91 g, $P < 0.01$) (Fig. 2).

3.5. Serum enzymological analyses

There were no significant differences in glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, albumin–globulin ratio, total protein, bilirubin, lactic dehydrogenase, alkaline phosphatase, γ -glutamyl transpeptidase and cholinesterase between the nicotine and the control group. There were also no differences in the plasma levels of triglyceride, total cholesterol and free fatty acid between the two groups (Table 1).

3.6. Histological examination of liver

Hematoxylin–eosin staining revealed hepatocytes and a fibroelastic structure that were not altered by nicotine administration (Fig. 3A,B).

The area of liver glycogen staining (periodic acid Schiff stain) from the nicotine group was significantly less than that from the control group, especially in the perivenous zone and periportal zone of the lobules (Fig. 3C,D).

3.7. Glycogen content, glycogen synthetase and phosphorylase-a activity

The glycogen content of the liver in the nicotine group was significantly lower than that in the control group (Fig. 4A). To elucidate the factor contributing to the glycogen content of the liver, the activity of glycogen synthetase and

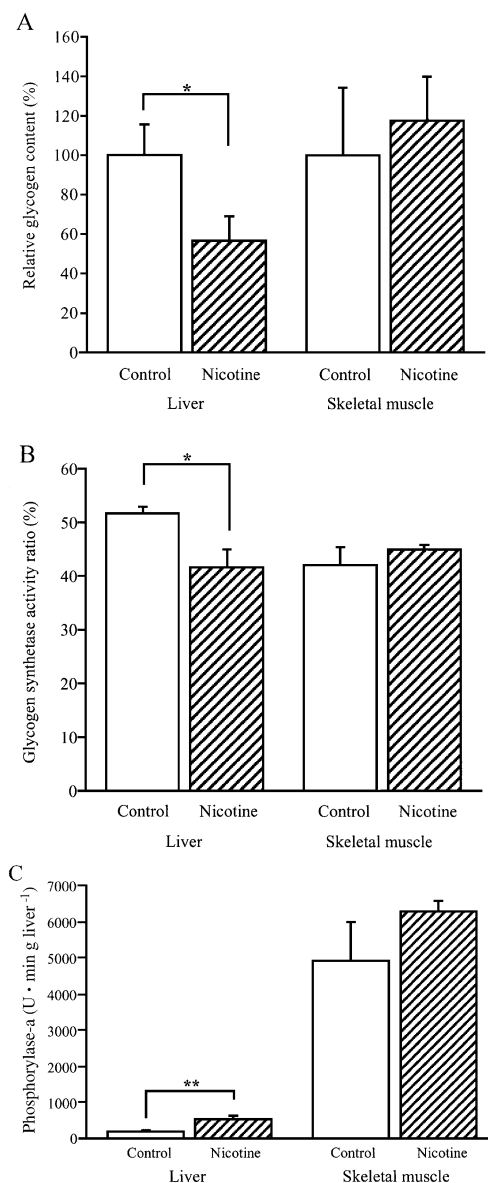


Fig. 4. Glycogen content (A), glycogen synthetase activity (B) and phosphorylase-a activity (C) of liver and skeletal muscle in nicotine and control groups. Means \pm S.E.M. (liver: $n = 7$; muscle: $n = 4$). * $P < 0.05$, ** $P < 0.01$ vs. control group. The ratio of the glycogen content as a percentage in liver from nicotine group to the percentage from control group liver was taken the relative glycogen content.

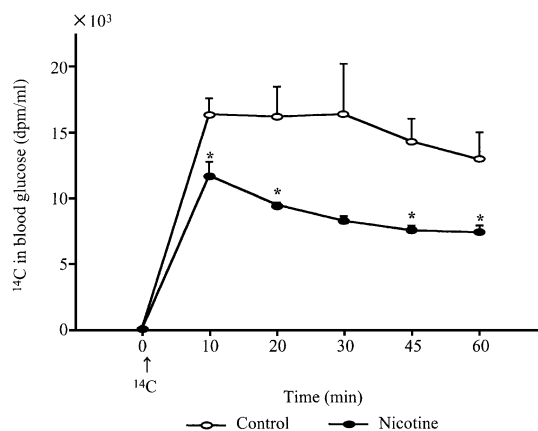


Fig. 5. Effects of nicotine on gluconeogenesis in nicotine and control groups. Values are the means \pm S.E.M. ($n=4$). * $P<0.05$ vs. control group.

phosphorylase-a was evaluated. Glycogen synthetase activity of the liver in the nicotine group was significantly lower than that in the control group ($41.90 \pm 2.90\%$ vs. $51.50 \pm 1.30\%$, $P<0.05$) (Fig. 4B). Phosphorylase-a activity of the liver in the nicotine group was significantly higher than that in the control group (715.45 ± 121.88 vs. 225.99 ± 85.38 U min g liver⁻¹, $P<0.05$) (Fig. 4C). On the other hand, glycogen content, glycogen synthetase, and phosphorylase-a activity of skeletal muscle were not significantly different between the nicotine and control group (Fig. 4A,B,C).

3.8. Gluconeogenic activity

Fig. 5 shows hepatic gluconeogenic activity in the nicotine group and in the control group. Gluconeogenesis was significantly lower in the nicotine group than in the control group 10, 20, 45 and 60 min after NaH¹⁴CO₃ administration. Total gluconeogenesis was significantly decreased in the nicotine group as compared with that in the control group ($483.92 \pm 16.88 \times 10^3$ vs. $843.66 \pm 115.06 \times 10^3$ dpm/ml, $P<0.05$) (Fig. 5).

4. Discussion

It has been reported that high concentrations of nicotine inhibit the insulin secretion induced by glucose, while low concentrations of nicotine stimulate insulin secretion in rabbit pancreas (Tjalve and Popov, 1973). There are several reports that smoking or subcutaneous injection of nicotine do not alter glucose tolerance or insulin action in type 2 diabetic patients (Epifano et al., 1992) or in adult male Sprague–Dawley rats in vivo (Swislocki et al., 1997). However, contradictory results also have been reported, i.e. that nicotine tends to decrease both basal and stimulated insulin secretions (Saah et al., 1994). On the other hand, smoking has been shown to impair insulin action in peripheral tissues and to lead to insulin resistance (Eliasson et al.,

1996). These discrepancies may be explained by a variety of factors, including the nutritional status, species difference, autonomic neural state, and the amount and route of administration of nicotine (Benowitz and Henningfield, 1994). The plasma nicotine levels in our experiments are comparable to those obtained from smokers in the human study (Attvall et al., 1993).

Oral nicotine administration was found to decrease significantly fasting blood glucose and the ratio of fasting insulin to glucose level, suggesting that nicotine may reduce insulin resistance in the obese rats in vivo (Liu et al., 2001). In the present study, plasma glucose levels after insulin load were significantly decreased after long-term oral nicotine administration, suggesting that nicotine may indeed reduce insulin resistance in the obese rats. Furthermore, we found that weight of the liver was significantly decreased after nicotine administration. We then examined serum transaminase and the histology of the liver. These results showed that hepatocytes were not damaged by nicotine either biochemically or histologically. We further measured the glycogen content, key regulatory enzyme activities involved in hepatic glycogen metabolism, as well as gluconeogenic activity. Our results showed that glycogen content per unit weight of liver decreased significantly on oral nicotine administration. Although phosphorylase-a activity was significantly increased, glycogen synthetase activity was significantly decreased by nicotine administration.

It has been reported that the autonomic system is involved in intracellular autoregulation of glycogen synthetase activity. Stimulation of α -adrenoceptors enhances glycogen synthesis while stimulation of β -adrenoceptors induces opposite changes (Dynarowicz, 1979). Nicotine increases significantly and dose dependently the plasma levels of catecholamines (Andersson et al., 1993; Grunberg et al., 1988). Recently, Andersson and Arner (2001) reported that systematically administered nicotine induces lipolysis, in part by activating the classical adrenergic mechanism. It is possible that in the present study high levels of catecholamines (β -adrenergic effect) caused by long-term oral nicotine administration led to a decrease of glycogen synthesis in the obese rats. It is also possible that the effect of nicotine is mediated directly through nicotine receptors in the liver to reduce glycogen synthesis (Dewar et al., 2002; Dynarowicz, 1979). Further studies are needed to clarify whether nicotine has a direct effect on hepatic glycogen content or has an effect through the autonomic system.

Syed and Khandelwal (2000) reported that insulin stimulates glycogen synthase in human liver cells (HepG2 cells) through the phosphatidylinositol-3 (PI-3) kinase pathway by activating protein kinase B and protein phosphatase-1 as well as inactivating glycogen synthase kinase-3 β . On the other hand, insulin inactivates phosphorylase activity through the PI-3 kinase pathway. In addition to the effect of nicotine on glycogenolysis, the present study showed that gluconeogenesis was significantly decreased after long-term nicotine administration. It has been reported that, in insulin-

resistant states, insulin no longer suppresses hepatic gluconeogenesis, and the consequent glucose overproduction contributes to hyperglycemia and diabetes mellitus (Kahn, 1994; Zhang et al., 2001). Although we cannot ascertain whether the decreased gluconeogenesis is due to a direct effect of nicotine or to lowered plasma insulin levels, the effect of nicotine to reduce insulin resistance can be explained in part by lowered gluconeogenesis.

Both muscle and liver glycogen reserves are important for whole body glucose homeostasis. The control values differ between muscle and liver partly due to the existence of different tissue-specific isoforms at key steps (Roach, 2002). In muscle, control of glycogen synthesis is shared between transport of glucose into the muscle and the step catalyzed by glycogen synthase. Muscle glycogen serves to fuel muscular activity and its utilization is controlled chiefly by muscle contraction and by catecholamines. Breakdown of liver glycogen, as part of blood glucose homeostasis, is also in response to nutritional cues. The differences in the effect of nicotine on insulin action in muscle and that in the liver in the present study may be explained by these tissue-specific regulations of glycogen metabolism.

Axelsson et al. (2001) reported that acute nicotine infusion impairs insulin sensitivity in type 2 diabetic patients. In contrast, the present study showed that long-term oral nicotine administration could increase insulin sensitivity in the obese diabetic rats, partly by decreasing hepatic glycogen content and also through gluconeogenesis, resulting in a decreased hepatic glucose release. The effect of nicotine on insulin sensitivity in muscle and adipose tissue remains to be elucidated.

Acknowledgements

This work was supported in part by a grant from the Smoking Research Foundation.

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