Brain-Derived Neurotrophic Factor Ameliorates Hepatic Insulin Resistance in Zucker Fatty Rats

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Brain-derived neurotrophic factor (BDNF), a member of the neurotrophins, has been reported to ameliorate hyperglycemia in obese diabetic animal models. To elucidate the mechanism of BDNF on glucose metabolism, we determined the glucose turnover under basal and euglycemic hyperinsulinemic (insulin infusion rate, 54 pmol · kg^{-1} · min^{-1}) clamp conditions in obese insulin-resistant rats, male Zucker fatty rats, which had been acutely administered a subcutaneous injection of BDNF (20 mg/kg) (n = 9, BDNF) or vehicle (n = 8, vehicle). Under the basal condition, acute administration of BDNF did not affect the blood glucose level, plasma insulin level, rate of glucose disappearance (Rd), and endogenous glucose production (EGP). Under the clamp condition, the glucose infusion rate (GIR) was significantly higher in BDNF than in vehicle (mean ± SD, 61.4 ± 19.1 *v* 41.4 ± 4.9 μmol · kg^{-1} · min^{-1}, *P* < .05). There was no significant difference in Rd and EGP between the 2 groups under the clamp condition, but the insulin-mediated suppression ratio of endogenous glucose production in BDNF was significantly greater than in vehicle (48.9 ± 22.2 *v* 22.4% ± 20.6%, *P* < .05). In BDNF, mRNA expressions of hepatic phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) were comparable to those of vehicle, while hepatic glucokinase (GK) mRNA expression was significantly higher (1.57 ± 0.33 *v* 1.03 ± 0.17, *P* < .05). We conclude that BDNF mainly improves hepatic insulin resistance in obese insulin-resistant rats, probably by affecting the hepatic GK flux.

BRAIN-DERIVED NEUROTROPHIC factor (BDNF), a member of the nerve growth factor-related neurotrophins, enhances the survival and differentiation of several classes of neurons in the central and peripheral nervous systems.[1,2] BDNF heterozygous knockout mice exhibit hyperphagia, and half of them become obese and insulin resistant.[3] Recent reports have shown that systemic administration of BDNF can markedly improve fasting hyperglycemia, hyperinsulinemia, and glucose intolerance in obese diabetic models, such as C57BL/KsJ-db/db mice and KK mice.[4-7] In streptozotocin (STZ)-induced diabetic mice, the glucose lowering effect of insulin was enhanced by both a large and a small amount of BDNF administered systemically and intracerebroventricularly, respectively.[7,8] These results suggest that BDNF can ameliorate hyperglycemia by improving insulin action, which may be implemented predominantly via the central nervous system.

Impaired insulin action is a common feature in obesity and type 2 diabetes.[9,10] Insulin resistance involves impaired glucose utilization in the peripheral tissues and elevated endogenous glucose production by the liver. In the present study, to clarify the effect of BDNF on glucose metabolism in these insulin-target organs, we evaluated peripheral and hepatic glucose fluxes using a euglycemic hyperinsulinemic clamp technique in an obese insulin-resistant model, Zucker fatty rats, with an acute administration of BDNF. Also, to determine the effect of BDNF on the liver, we measured the mRNA expressions of key enzymes of glucose efflux in the liver, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), as well as the mRNA expression and activity of glucokinase (GK), a key enzyme of glucose influx in the liver.

MATERIALS AND METHODS

Animals and Catheterization

Male Zucker fatty rats were purchased from Japan SLC (Hamamatsu, Japan). The animals were allowed free access to water and standard laboratory rat chow (Clea Japan, Tokyo, Japan), and were individually housed under controlled temperature (23°C) and lighting (12-hour light, 8 AM to 8 PM; 12-hour dark, 8 PM to 8 AM). Rats, weighing 448 ± 24 g, were used for the experiment at 16 to 17 weeks of age. All procedures were conducted according to the guidelines of the Sumitomo Pharmaceuticals Committee on Animal Research. Four days before the study, a silicon catheter (Phicon Tube; Fuji-Systems, Tokyo, Japan) was inserted into the right jugular vein for infusion, and a polyethylene catheter (PE-50; Clay Adams, Sparks, MD) was inserted into the left carotid artery for blood sampling under general anesthesia with sodium pentobarbital.[11,12]

Experimental Protocol

Studies were performed on rats under conscious and unstressed conditions after an overnight fast. The rats were used in the experiments only if they had a good appetite and less than 10% body weight loss after surgery. Rats were divided into 2 study groups and injected with 20 mg/kg human recombinant BDNF (NH_2-terminal methionine-free) from Regeneron Pharmaceuticals (Tarrytown, NY) (n = 9) or 0.01% Tween 80 and 1% mannitol in phosphate-buffered saline (PBS) (vehicle) (n = 8) subcutaneously according to our previous reports.[6] Each experiment consisted of a 90-minute tracer equilibration period (from -120 to -30 minutes), a 30-minute basal sampling period (from -30 to 0 minutes, basal period), and a 150-minute euglycemic hyperinsulinemic clamp period (from 0 to 150 minutes, clamp period) (Fig 1).

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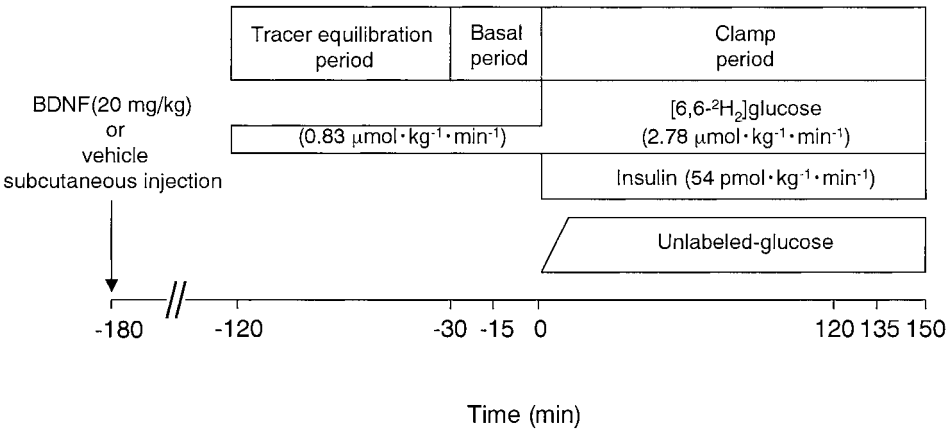

Fig 1. Experimental protocol.

At -180 minutes, BDNF or vehicle was injected subcutaneously to each group of rats. A priming infusion (8.33 μmol · kg^{-1} · min^{-1} over 10 minutes) followed by a continuous infusion (0.83 μmol · kg^{-1} · min^{-1}) of [6,6-$^2H_2$]glucose (Cambridge Isotope Laboratories, Andover, MA) was started through the jugular vein at -120 minutes. To prevent a decrease in the enrichment during the euglycemic hyperinsulinemic clamp, the infusion rate of [6,6-$^2H_2$]glucose was increased to 2.78 μmol · kg^{-1} · min^{-1} at 0 minute and continued until 150 minutes. At 0 minute, a priming infusion (540 pmol · kg^{-1} · min^{-1} over 5 minutes) followed by a constant infusion (54 pmol · kg^{-1} · min^{-1}) of regular human insulin (Eli Lilly, Indianapolis, IN) was started and continued during the clamp period. During this period, the arterial blood glucose level was monitored every 5 minutes, and the rate of glucose (20% dextrose) infusion into the jugular vein was adjusted to maintain arterial blood glucose levels at the fasting levels as described by Kraegen et al.[13] At the end of the experiment, the rats were anesthetized with an intravenous injection of pentobarbital, the abdomen was quickly opened, and the liver was freeze-clamped and stored at -80°C until the assay.

Blood samples of 200 μL were taken at -120, -30, -15, 0, 120, 135, and 150 minutes to determine the isotopic enrichment of [6,6-$^2H_2$]glucose. To measure plasma insulin, free fatty acids (FFA), and glucagon levels, 900-μL blood samples were taken at -120, 0, and 150 minutes. To measure plasma glucagon and cathecolamine levels, 1,500-μL blood samples were taken at 150 minutes. The prechilled tubes for FFA and glucagon contained EDTA with a lipoprotein lipase inhibitor, diethyl p-nitrophenyl phosphate (Sigma, St Louis, MO) and aprotinin (FBA Pharmaceuticals, New York, NY), respectively.

Analytical Procedures

Blood glucose levels were determined using a blood glucose meter (Hemocue, Mission Viejo, CA).[14] Plasma rat insulin concentration was measured at -120 and 0 minutes by enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi, Gunma, Japan). Plasma human insulin concentration was measured at 150 minutes using a double antibody solid-phase technique radioimmunoassay kit. The cross-reactivity of rat insulin is approximately 30% in this assay. The plasma glucagon level was measured using an enzyme immunoassay kit (Yanaihara Institute, Shizuoka, Japan). Plasma FFAs were determined by the enzymatic method.[15] Plasma BDNF was assayed by enzyme-linked immunoassay as described previously.[16] Plasma cathecolamines were assayed using high-performance liquid chromatography.

Glycogen was extracted from the liver using amyloglucosidase according to the method of Lust et al.[17] The glucose derived from glycogen was determined using the glucose oxidase method.

The enrichment of plasma [6,6-$^2H_2$]glucose was determined as described previously.[11,12] In brief, after deproteinization by 99.5% ethanol, the plasma sample was derivatized by N-methyl-bis-trifluoroacetamide (Pierce, Rockford, IL), and trifluoro-acetylated glucose was measured using a gas chromatography-mass spectrometer (Model TSQ-700; Finningan-MAT, San Jose, CA) with a silicon SE-30 capillary column (Gasukuro Kogyo, Tokyo, Japan).

Calculations of Glucose Fluxes

The rates of glucose appearance (Ra) and disappearance (Rd) were calculated according to Steele's nonsteady-state equations.[18] Endogenous glucose production (EGP) was calculated as the difference between the tracer-derived Ra and exogenous infusion rates of glucose (GIR) and tracer. The insulin-mediated increase ratio of Rd (%ΔRd) was calculated by the following equation:

$$\%\Delta Rd = (Clamp\ Rd - Basal\ Rd)/\ Basal\ Rd \times 100\ (\%)$$

in which Basal Rd and Clamp Rd are the Rd in the basal and the clamp period, respectively. The insulin-mediated suppression ratio of EGP (%ΔEGP) was calculated by the following equation:

$$\%\Delta EGP = (Basal\ EGP - Clamp\ EGP)/Basal\ EGP \times 100\ (\%)$$

in which Basal EGP and Clamp EGP are the EGP in the basal and the clamp period, respectively. The results are represented as the average of metabolic parameters from -30 to 0 minutes in the basal period and from 120 to 150 minutes in the clamp period.

Northern Blotting Analyses

Total RNA was extracted from the liver. Northern blots followed standard procedures using 20 μg total RNA, and the buffer conditions were as described. Reverse transcription-PCR was performed using primers [sense 5′-TGGTCTGGACTTCTCTGCCAAG-3′ and antisense 5′-ACCGTCTTGCTTTCGATCCTGG-3′] for PEPCK (PCR product, 258 bp), [sense 5′-TGTCTTGGTGTCTGTGATCGCTG-3′ and antisense 5′- AAGTGAGCCGCAAGGTAGATCC-3′] for G6Pase (PCR product, 441 bp), and [sense 5′-TGCAGAAGGAGATGGACCGT-3′ and antisense 5′-CAGGGAAGGAGAAGGTGAAG-3′] for GK (PCR product, 351 bp). The probed membranes were exposed to an imaging plate, BAS-MS 2040 (Fujifilm, Tokyo, Japan). The hybridization intensity was quantified using a BAS2500 system (Fujifilm, Tokyo, Japan). To correct the loading differences of RNA, each membrane was reprobed with mouse β actin cDNA probe.[19]

Table 1. Blood Glucose, Plasma Rat Insulin, Plasma Human Insulin, Plasma Glucagon, Cathecolamine, FFA, and BDNF

	−120 Minutes		0 Minute		150 Minutes	
	BDNF	Vehicle	BDNF	Vehicle	BDNF	Vehicle
Glucose (mmol/L)	6.4 ± 0.8	6.4 ± 0.4	6.4 ± 0.4	6.4 ± 0.7	6.5 ± 0.5	6.3 ± 0.5
Rat insulin (pmol/L)	1,114 ± 340	1,281 ± 377	1,357 ± 729	1,257 ± 841		
Human insulin (pmol/L)					1,627 ± 337*	2,048 ± 464
Glucagon (ng/mL)			0.57 ± 0.26	0.41 ± 0.10	1.28 ± 0.83	1.16 ± 0.82
Epinephrine (pg/mL)					90.5 ± 111.6	68.8 ± 45.7
Norepinephrine (pg/mL)					278.2 ± 97.7	236.3 ± 85.2
FFA (mEq/L)	0.89 ± 0.36	0.74 ± 0.18	0.74 ± 0.18	0.74 ± 0.18	0.48 ± 0.20†	0.45 ± 0.17†
BDNF (ng/mL)	4,084 ± 3,221	ND	1292 ± 860‡	ND	779 ± 295§	ND

NOTE. Data are expressed as mean ± SD.

Abbreviation: ND, not detectable.

*$P < .05$ *v* vehicle group by Student's *t* test.

†$P < .01$ *v* values at −120 minutes and 0 minute by Student's *t* test.

‡$P < .05$ *v* values at −120 minutes by Student's *t* test.

§$P < .01$ *v* values at −120 minutes by Student's *t* test.

Hepatic GK Activity

Hepatic GK activity was determined using a modification of a previously reported spectrophotometric method.[12, 20]

Statistical Analysis

Data are represented as mean ± SD. Statistical comparisons between the BDNF and vehicle groups were assessed using Student's 2-tailed unpaired *t* test. Comparisons of repeated measurements within each experimental group were assessed using analysis of variance (ANOVA) or Student's 2-tailed paired *t* test where appropriate. Statistical significance was accepted at $P < .05$.

RESULTS

Basal Period

At -120 minutes, arterial blood glucose, plasma insulin, and FFA levels were similar between the BDNF and vehicle groups (Table 1). During the basal period, the arterial blood glucose, plasma insulin, glucagon, and FFA levels were comparable between the 2 groups. Rd and EGP were similar between the 2 groups (Figs 2 and 3).

Clamp Period

During the clamp period, stable and comparable arterial blood glucose levels were achieved in the BDNF and vehicle groups (BDNF, 6.5 ± 0.5; vehicle, 6.3 ± 0.5 mmol/L) (Fig 4). The arterial plasma human insulin level at 150 minutes was significantly lower in the BDNF group than in the vehicle group ($P < .05$) (Table 1). The plasma BDNF level decreased gradually during the experiment in the BDNF group. Arterial plasma glucagon, cathecolamines, and FFA levels were comparable between the 2 groups at 150 minutes. Plasma FFA levels during the clamp period were significantly lower in both groups compared with -120 minutes, and the basal period ($P < .05$). GIR was similar between the 2 groups from 0 to 60 minutes and significantly higher in the BDNF group than in the vehicle group from 95 to 150 minutes (Fig 4). The average GIR from 120 to 150 minutes was 61.4 ± 19.1 and 41.4 ± 4.9 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ ($P < .05$) in the BDNF and the vehicle groups, respectively. Rd during the clamp period was comparable between the 2 groups (BDNF, 94.4 ± 17.5; vehicle, 83.4 ± 17.1 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) (Fig 3). EGP tended to be lower in the BDNF group than in the vehicle group (BDNF, 30.4 ± 13.8; vehicle, 39.2 ± 15.1 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) (Fig 4). %ΔRd did not differ between the 2 groups (BDNF, 58.5 ± 40.2; vehicle, 66.2% ± 26.0%) (Fig 2), however, %ΔEGP was significantly greater in the BDNF group than in the vehicle group (BDNF, 48.9 ± 22.2; vehicle, 22.4% ± 20.6%; $P < .05$) (Fig 3).

Hepatic Glycogen Content

The glycogen content of the liver isolated at the end of the clamp period was not significantly different between the 2 groups (BDNF, 23.7 ± 5.9; vehicle, 24.5 ± 9.0 μg/mg protein).

mRNA Expression and Activity of Hepatic Enzymes

To further investigate the cellular action of BDNF in the liver, we measured the mRNA levels of the key enzymes of glucose metabolism, which is regulated by insulin. PEPCK mRNA expression was comparable between the 2 groups (BDNF, 0.35 ± 0.05; vehicle, 0.36 ± 0.19 arbitrary units). G6Pase mRNA expression was also comparable between the 2 groups (BDNF, 0.25 ± 0.22; vehicle, 0.22 ± 0.08 arbitrary units). GK mRNA expression was significantly higher in the BDNF group than in the vehicle group (BDNF, 1.57 ± 0.33; vehicle, 1.03 ± 0.17; $P < .05$) (Fig 5). Hepatic GK activity was 33% higher in the BDNF group than in the vehicle group, but the difference was not significant (BDNF, 15.6 ± 5.0; vehicle, 11.7 ± 3.6 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$) (Fig 5).

DISCUSSION

In the present study, acute administration of BDNF was found to ameliorate insulin resistance in obese insulin-resistant Zucker fatty rats. Insulin resistance is explained as a decrease in the ability of insulin to stimulate glucose disposal in the peripheral tissues and to suppress endogenous glucose production.[9,10] We found that the effect of BDNF on insulin resistance is mainly due to improvement of insulin-mediated suppression of endogenous glucose production. Under the euglycemic hy-

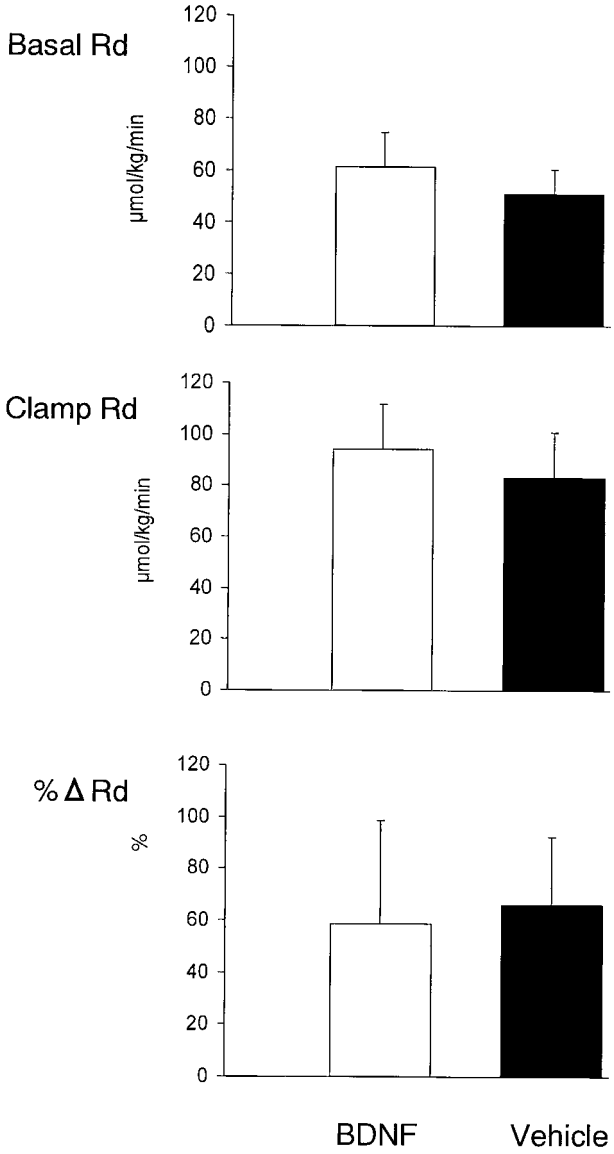

Fig 2. Rates of Rd in the basal period (basal Rd) and in the clamp period (clamp Rd) and insulin-mediated increase ratio of Rd (%ΔRd) in Zucker fatty rats with (□) and without (■) BDNF treatment. Data are expressed as mean ± SD.

perinsulinemic clamp condition, endogenous glucose is mainly produced from the liver,[21,22] which may therefore be the main target organ of BDNF. A single injection of BDNF has been reported to enhance insulin-stimulated tyrosine phosphorylation of insulin receptors and phosphatidyl inositol 3-kinase activity in the liver, but not in the skeletal muscle.[8] This finding is consistent with the present in vivo result.

BDNF is secreted by neurons and other types of brain cells and is considered to act in a paracrine or autocrine manner.[23] In particular, the expression of BDNF is high in the hypothalamus, the neural center of glucose metabolism.[24] TrkB, the specific receptor of BDNF, is also richly expressed in the hypothalamus, but is not expressed in the liver, muscle, and adipose tissue.[25,26] In previous reports, a small amount (≃ 0.25 mg/kg) of BDNF injected into the third ventricle showed a hypoglycemic effect comparable to 20 mg/kg of BDNF injected subcutaneously in STZ-induced diabetic mice.[7,8] In addition, no direct effect of BDNF on glucose release and insulin signaling has been found in primary cultured hepatocytes.[8] These findings suggest that BDNF ameliorates insulin resistance probably through the central nervous system.

Hepatic glucose production is mediated by neural regulation in both a direct and an indirect manner. The liver has an ample supply of autonomic nerves with signals originating in the hypothalamus. We have reported that innervation of the hepatic vagus nerve directly regulates hepatic glucose production and uptake.[12] On the other hand, the nervous system, including the central and autonomic nervous systems, can influence hepatic

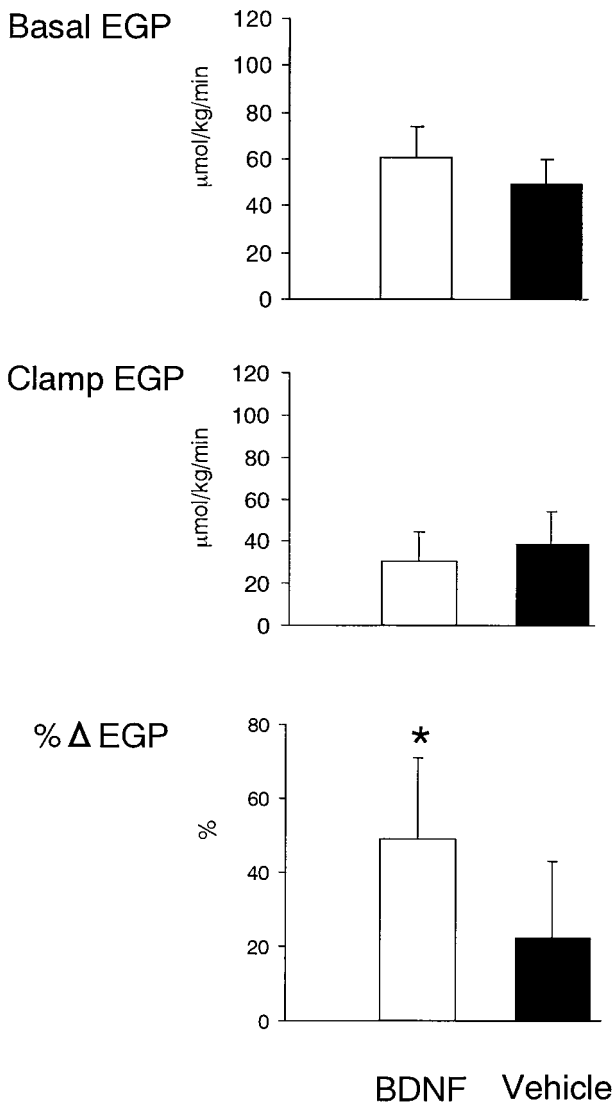

Fig 3. EGP in the basal period (basal EGP) and in the clamp period (clamp EGP) and insulin-mediated suppression ratio of EGP (%ΔEGP) in Zucker fatty rats with (□) and without (■) BDNF treatment. Data are expressed as mean ± SD. *$P < .05$ *v.* vehicle group.

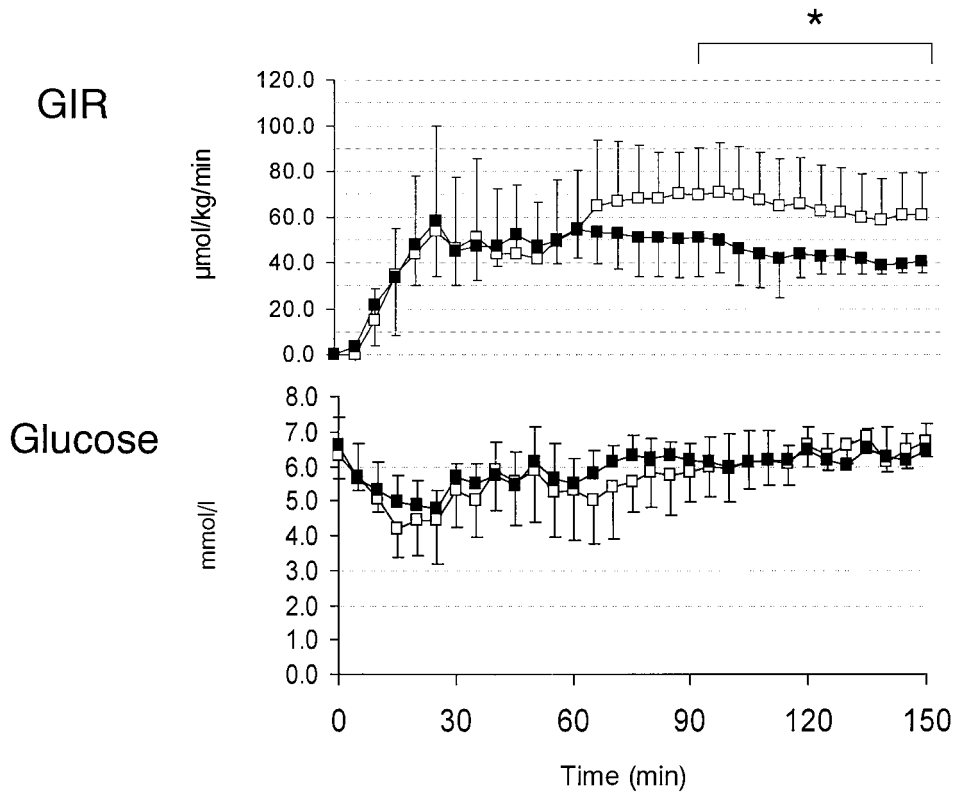

Fig 4. GIR and blood glucose level (glucose) in Zucker fatty rats with (□) and without (■) BDNF treatment during euglycemic hyperinsulinemic clamp. Data are expressed as mean ± SD. *$P < .05$ *v* vehicle group.

glucose production indirectly by altering various hormones and substrates, such as glucagon, cathecolamines, and FFA. However, we found that BDNF did not affect hormones and substrate levels. Therefore, we suggest that BDNF may enhance insulin action in the liver by affecting the central and autonomic nervous system.

Hepatic glucose production consists of positive fluxes of glycogenolysis and gluconeogenesis and a negative flux of

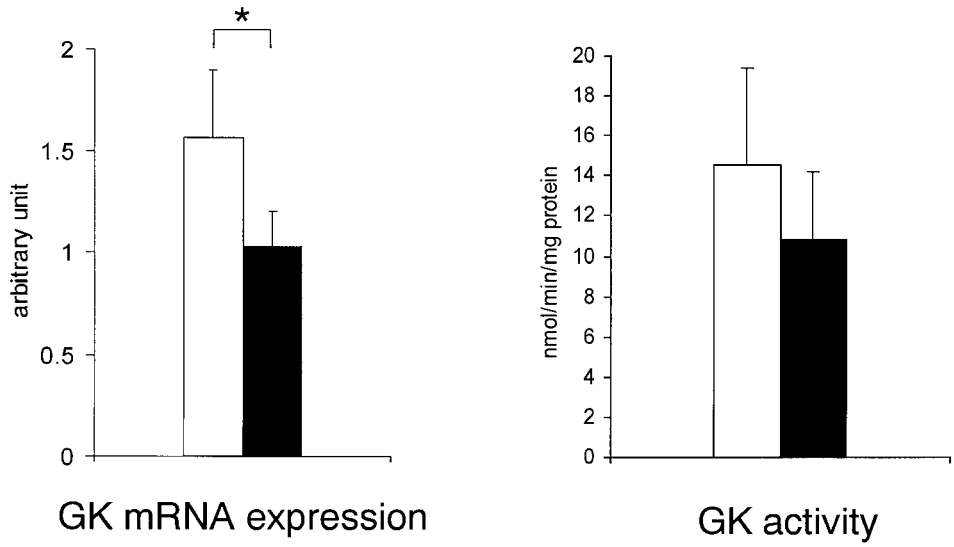

Fig 5. mRNA expression and activity of hepatic GK in Zucker fatty rats with (□) and without (■) BDNF treatment. Data are expressed as mean ± SD. *$P < .05$ *v* vehicle group.

GK.[20,21] BDNF did not affect the hepatic glycogen content and mRNA expressions of PEPCK and G6Pase, but it enhanced GK mRNA expression and tended to increase GK activity. These findings suggest that BDNF ameliorates hepatic insulin resistance by stimulating GK flux, but not glycogenolytic and gluconeogenic fluxes in the liver. A recent study in hepatic GK transgenic mice showed that a modest increase in hepatic GK activity reduced fasting and fed plasma glucose levels at even lower insulin concentration compared with non-transgenic mice.[27] Thus, increased GK flux can ameliorate insulin sensitivity by reducing hepatic glucose production.

In the present study, plasma human insulin concentration was significantly lower in the BDNF group than in the vehicle group. This suggested that BDNF increased insulin clearance in obese insulin-resistant rats. With such a lower insulin level, insulin-stimulated peripheral glucose utilization might be underestimated in BDNF-treated rats. Therefore, BDNF could enhance insulin action in the peripheral tissues, as well as the liver. However, because insulin-suppressed endogenous glucose production was also underestimated, improved hepatic insulin action would have mainly contributed to an increased glucose infusion rate (GIR) under the euglycemic hyperinsulinemic condition.

In conclusion, BDNF mainly improves hepatic insulin resistance in obese insulin-resistant rats, probably affecting the hepatic GK flux.

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