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JTT-501, a novel oral antidiabetic agent, improves insulin resistance in genetic and non-genetic insulin-resistant models

[1,2]Tsutomu Shibata, [1]Kenichi Matsui, [1]Fumihiko Yonemori & [1]Korekiyo Wakitani

[1]Japan Tobacco, Inc., Central Pharmaceutical Research Institute, 1-1, Murasaki-cho, Takatsuki, Osaka, 569-1125, Japan

1 We investigated whether JTT-501 (4-[4-[2-(5-methyl-2-phenyl-4-oxazolyl)ethoxy]benzyl]-3,5-isoxazolidinedione) would improve insulin resistance in genetic (Zucker fatty rats) and non-genetic (high-fat fed rats) rodent models of obesity.

2 JTT-501 (10–100 mg kg^{-1} day^{-1}) was administered orally to Zucker fatty rats for 7–21 days. In the high-fat fed rat model, JTT-501 (100 mg kg^{-1} day^{-1}) was administered orally for 7 days. In both models, JTT-501 improved metabolic abnormalities by enhancing insulin action during the glucose tolerance test and the euglycaemic-hyperinsulinaemic clamp study. In *ex vivo* assays, JTT-501 ameliorated the impaired insulin-sensitive glucose oxidation and lipid synthesis in peripheral tissues. Furthermore, JTT-501 enhanced insulin receptor autophosphorylation in hindlimb muscle.

3 JTT-501 reduced serum leptin concentrations in both models, but did not affect body weight or epididymal fat weight.

4 Our observations indicate that JTT-501 improves the metabolic abnormalities in both genetic and non-genetic insulin-resistant models by enhancing insulin action in peripheral tissues. These effects of JTT-501 are due, at least in part, to enhanced insulin receptor autophosphorylation. In addition, JTT-501 is able to reduce serum leptin concentrations in hyperleptinaemia of the insulin-resistant model. We expect JTT-501 to show promise for treating non-insulin dependent diabetes mellitus patients with insulin resistance.

Keywords: JTT-501; isoxazolidinedione; insulin resistance; NIDDM; insulin receptor; leptin

Abbreviations: BSA, bovine serum albumin; ECL, enhanced chemiluminescence; EDTA, ethylenediamine-N,N,N′,N′-tetraacetic acid; GIR, glucose infusion rate; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid; IR index, insulin resistance index; IRS-1, insulin receptor substrate-1; NIDDM, non-insulin dependent diabetes mellitus; PI 3-kinase, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulphate; TZDs, thiazolidinediones

Introduction

In non-insulin dependent diabetes mellitus (NIDDM) and obesity, insulin resistance is a characteristic feature and is caused by insulin receptor and/or post-receptor signalling defects (Maegawa *et al*., 1991; Grasso *et al*., 1995; Goodyear *et al*., 1995; Kahn, 1994). The relationship between insulin resistance and insulin signalling defects is also supported by studies using knockout mice (Araki *et al*, 1994; Tamemoto *et al*., 1994; Brüning *et al*., 1997). Although a multitude of environmental and genetic factors participate in the insulin signalling defects, the main etiological factors have not yet been established (Taylor, 1989; O'Doherty *et al*., 1997). In addition, insulin resistance appears to be a syndrome that is associated with a clustering of metabolic disorders such as NIDDM, obesity, hypertension, lipid abnormalities, and atherosclerotic cardiovascular disease (Reaven, 1988; DeFronzo & Ferrannini, 1991). In light of this evidence, it is very important to develop the appropriate therapy for insulin resistance as a multiple risk factor.

Recently, the thiazolidinediones (TZDs) have been developed as new oral antidiabetic agents, insulin sensitizers, for NIDDM patients in order to enhance insulin sensitivity in muscle, adipose tissue and liver, and to improve insulin resistance in these target sites (Saltiel & Olefsky, 1996; Fujiwara *et al*., 1988; Sugiyama *et al*., 1990; Young *et al*., 1995; Stevenson *et al*., 1990). As a result of screening for insulin sensitizing agent, we found JTT-501 (4-[4-[2-(5-methyl-2-phenyl-4-oxazolyl)ethoxy]benzyl]-3,5-isoxazolidinedione) which is structurally distinct from the TZDs (Shinkai *et al*., 1988). In this report, we examined and characterized the effects of JTT-501 on insulin resistance in Zucker fatty rats and high-fat fed rats, as models of genetic and non-genetic obese/insulin resistance, respectively.

Methods

Animals

These experiments complied with the Guidelines of Animal Experimentation of our laboratories. Male Zucker fatty (fa/fa) and lean (Fa/+) rats were purchased from Charles River Japan, Inc. (Tokyo, Japan). Male Sprague-Dawley rats were purchased from Keali Co. Ltd. (Osaka, Japan). Animals were maintained on standard laboratory chow diet (3.6 kcal g^{-1}; carbohydrate 53.5%, protein 23.1%, and fat 5.9%, Oriental Yeast, Tokyo, Japan), and water *ad libitum*. High-fat fed rats (from 5 weeks old) were maintained on a high-fat diet (6.7 kcal g^{-1}; carbohydrate 7.5%, protein 24.5%, and fat 60%, Oriental Yeast) for 2–3 weeks. They were housed in plastic cages in a room controlled for temperature (25±3°C), humidity (55±15%), and light (0800–2000 h).

Compound and administration

JTT-501 (4-[4-[2-(5-methyl-2-phenyl-4-oxazolyl)ethoxy]benzyl]-3,5-isoxazolidinedione) was synthesized by the Japan

[2]Author for correspondence.

Tobacco, Inc., Central Pharmaceutical Research Institute. JTT-501 was suspended in 0.5% sodium carboxymethylcellulose (0.5% CMC-Na, Tokyo Kasei, Tokyo, Japan) solution, and was administered orally to rats once daily by stomach tube. Zucker fatty rats (10–12 weeks old) received JTT-501 (10–100 mg kg^{-1} day^{-1}) for 7–21 days. High fat fed rats (5 weeks old) received the high-fat diet for 2–3 weeks and were treated with JTT-501 (100 mg kg^{-1} day^{-1}) for the last 7 days of the diet period. Matched control and normal rats were treated with 0.5% CMC-Na solution for this period.

Analytical method

The serum glucose and triglyceride concentrations were determined using a commercial kit (Boehringer Mannheim, Tokyo, Japan) by COBAS FARA II (Roche, Tokyo, Japan). The serum insulin concentrations were determined using an insulin radio immunoassay kit (Shionogi, Tokyo, Japan) with rat insulin (Linco Research, St. Charles, MO, U.S.A.) as a standard by automatic gamma counter 1470 WIZARD™ (Wallac Oy, Turuk, Finland). The serum leptin concentrations were determined using a rat leptin enzyme-linked immunosorbent assay kit (Amersham, Buckinghamshire, U.K.) using a microplate reader THERMO max (Molecular Devices, Menlo Park, CA, U.S.A.). The minimum detectable concentration of the insulin and leptin assays were 0.1 and 0.2 ng ml^{-1}, respectively, and the intra- and inter-assay variations were less than 5%.

Glucose loading test

Rats were fasted for 16 h after the 7th administration of JTT-501. D-glucose solution (2 g kg^{-1}) was given orally to rats, and blood samples were taken from the tail vein at 15 to 60 min intervals thereafter. These samples were used for measuring serum glucose and insulin concentrations. Areas under the glucose and insulin concentration time curves for 0–120 min were determined by multiplying cumulative mean height of the glucose values (1 mmol l^{-1} = 1 cm) and insulin values (1 pmol l^{-1} = 1 cm), respectively, by time (60 min = 1 cm) and were defined as glucose area and insulin area, respectively. The insulin resistance (IR) index was determined to compare the resistance to insulin action between the groups, and was derived from the product of the glucose and insulin curves (Mondon *et al*., 1981; Mukherjee *et al*., 1997). A large IR index reflects elevated insulin resistance, indicating insulin is less effective in lowering serum glucose levels.

Glucose oxidation and total lipid synthesis in adipose tissues

Epididymal fat pads of Zucker rats were removed at 24 h after the last administration of JTT-501. Adipose tissue samples were incubated in Hanks' balanced salt solution (pH 7.4, GIBCO, Grand Island, NY, U.S.A.) containing 4% BSA (bovine serum albumin, Sigma Chemical, St. Louis, MO, U.S.A.), 20.72 kBq ml^{-1} [U]-^{14}C-glucose (NEN, Boston, MA, U.S.A.), and 0–100 mU ml^{-1} insulin (from bovine pancreas, Sigma) for 120 min at 37°C. Synthesized $^{14}CO_2$ was trapped with Scintilamine®-OH (Dojindo, Tokyo, Japan) on filter paper (Whatman, Maidstone, U.K.) and counted using a Liquid Scintillation Analyzer Model 2500 TR (Packard, Meriden, CT, U.S.A.). Total lipid synthesis from glucose was estimated by determining the incorporation of ^{14}C into total lipids. These were extracted from the tissues with n-hexane after the addition of an aliquot of Dole's solution (2-propanol : n-heptane : 1 N H_2SO_4 = 40 : 10 : 1). Radioactivity in the hexane fractions was measured using a liquid scintillation counter.

Euglycaemic-hyperinsulinaemic clamp study

Rats were fasted for 16 h after the seventh administration of JTT-501. Cannulas were implanted in the left carotid artery and right jugular vein under pentobarbital anaesthesia (50 mg kg^{-1}, Nembutal, Dynabbott, Osaka, Japan), and exteriorized through the back of the neck. These lines were kept patent by filling with sodium heparin solution (100 U ml^{-1} in saline, Midorijuji, Osaka, Japan). The euglycaemic-hyperinsulinaemic clamp was performed in the conscious state. Human regular insulin (Novo Nordisk, Copenhagen, Denmark) was infused at a constant rate (14 mU kg^{-1} min^{-1}) *via* the venous cannula for 150 min. Blood samples (50 μl) were taken from the arterial cannula for measurement of blood glucose concentrations by a blood glucose analyzer (Mediace GR-100) (Terumo, Tokyo, Japan), at 5 min intervals. Blood glucose concentration was maintained constant at basal feeding concentrations (approximately 6.1 mmol 1^{-1}) by a variable rate glucose infusion *via* the venous cannula. When blood glucose stabilized within 6.1 ± 0.6 mmol 1^{-1} for 30 min and glucose infusion rate was further maintained constant during constant insulin infusion, we defined this condition as the steady state. In the steady state, blood samples (200 μl) were taken from the arterial cannula at the final two points (145 and 150 min) for measurement of serum glucose and insulin concentrations.

Autophosphorylation of the insulin receptor

Zucker rats were injected intravenously with human regular insulin (5 U kg^{-1}, Novo Nordisk) or saline into the tail vein. The hindlimb muscles were removed at 5 min after insulin injection and quickly frozen in liquid N_2. Muscles were homogenized in ice-cold lysis buffer containing 50 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid, pH 7.4), 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 4 mM EDTA (ethylenediamine-N,N,N′,N′-tatraacetic acid), 2 mM sodium orthovanadate, 1% Triton X-100, 2 mM PMSF (phenylmethylsulphonyl fluoride), and 100 μg ml^{-1} aprotinin. Homogenates were allowed to solubilize on ice for 1 h. Insoluble material was removed by centrifugation at $12{,}000 \times g$ at 4°C for 30 min. Supernatants containing equal amounts of total muscle protein (10 mg) were immunoprecipitated overnight with anti-insulin receptor monoclonal antibody Ab-3 (Oncogene, Cambridge, MA, U.S.A.). Immune complexes were collected with protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden), washed, solubilized in Laemmli sample buffer and separated using 6% SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis (TEFCO, Tokyo, Japan). Proteins were transferred from gel to PVDF (polyvinylidene difluoride) membranes (BIO RAD, Richmond, CA, U.S.A.) using a semidry transfer apparatus (BIO RAD). Immunoblotting was performed with anti-phosphotyrosine monoclonal antibody 4G10 (UBI, Lane Placid, NY, U.S.A.), followed by horseradish peroxidase conjugated anti-mouse IgG antibody (Amersham) and detected with the ECL (enhanced chemiluminescence) system (Amersham). Band intensities were quantified by a Model GS-700 Imaging Densitometer (BIO RAD).

Statistical analysis

The results were presented as the means ± s.e.mean. Significance of differences between two groups was evaluated using

Student's *t*-test. For multiple comparisons, one-way analysis of variance (ANOVA) was used. When ANOVA showed significant differences, *post-hoc* analysis was performed with Dunnett's test or Tukey test. Significances of time course data (Figure 1) were determined by two-way (treatment and time) repeated measure ANOVA analysis of variance. If there was a significant difference, one-way (treatment) ANOVA followed by Dunnett's test for comparison to control group was performed, $P<0.05$ was considered statistically significant.

Results

Effects of JTT-501 on metabolic abnormalities

Genetic obese/insulin-resistant Zucker fatty rats showed hyperglycaemia ($F(1.8)=18.313$, $P=0.0027$, Figure 1A), hyperinsulinaemia ($F(1.8)=29.385$, $P=0.0006$, Figure 1B) and hypertriglyceridaemia ($F(1, 8)= 24.637$, $P=0.0011$, Figure 1C) compared with lean rats. Oral administration of JTT-501 (10,30 and 100 mg kg^{-1} day^{-1}) reduced serum triglyceride concentrations (factor treatment: $F(3,16)=11.427$, $P=0.0003$, factor time: $F(4,64)=15.848$, $P=0.0001$, interaction: $F(12,64)=2.384$, $P=0.0130$) and tended to decrease serum glucose concentrations (factor treatment: $F(3,16)=2.882$, $P=0.0683$, factor time: $F(4,64)=1.140$, $P=0.3458$, interaction: $F(12,64)=1.121$, $P=0.3598$) and serum insulin concentrations (factor treatment: $F(3,16)=1.814$, $P=0.1853$, factor time: $F(4,64)=6.092$, $P=0.0003$, interaction: $F(12,64)=1.182$, $P=0.3147$).

Fasting glucose and insulin concentrations of high-fat fed rats were significantly increased compared with control rats (Figures 2A and B). JTT-501 (100 mg kg^{-1} day^{-1}) treatment decreased fasting glucose and insulin concentrations to the levels of control rats.

Effect of JTT-501 on glucose tolerance

In the glucose tolerance test, augmentation of the glucose and insulin curve area in Zucker fatty rats was higher than that of

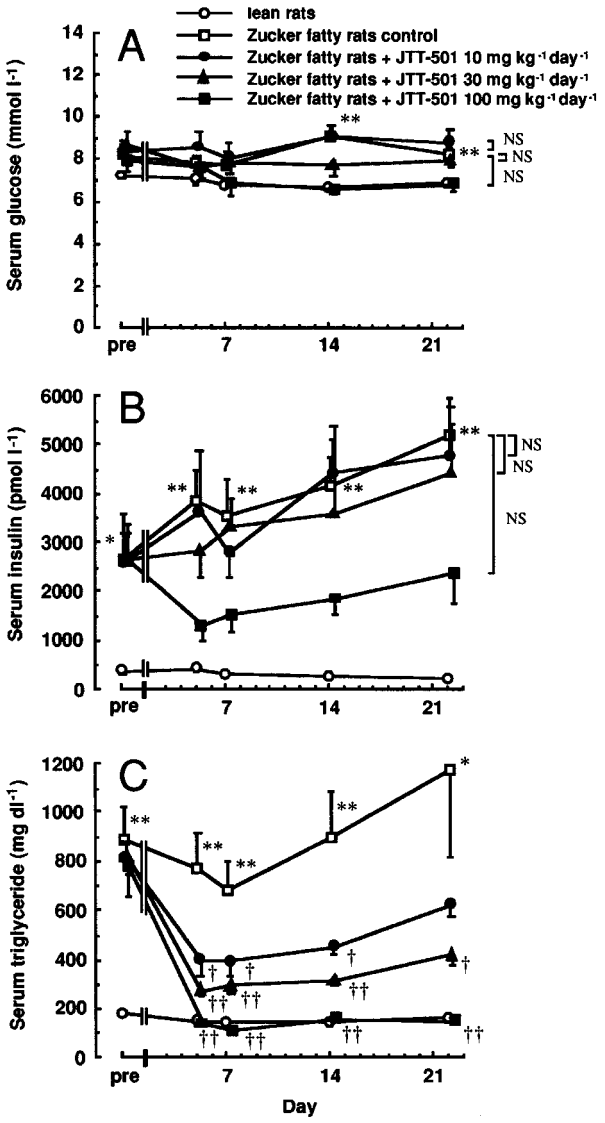

Figure 1 Effects of JTT-501 on serum glucose, insulin and triglyceride concentrations in Zucker fatty rats. JTT-501 (10, 30 and 100 mg kg^{-1} day^{-1}) was orally administered to Zucker fatty rats for 21 days. Blood samples were taken from the tail vein at 24 h after the 4th, 6th, 13th, and 21st administration. Each point represents the means±s.e.mean ($n=5$). *$P<0.05$, **$P<0.01$ vs lean rats (Student's *t*-test), †$P<0.05$, ††<0.01 vs Zucker fatty rats control (Dunnett's test). NS, not significant vs Zucker fatty rats control (two-way repeated measure ANOVA).

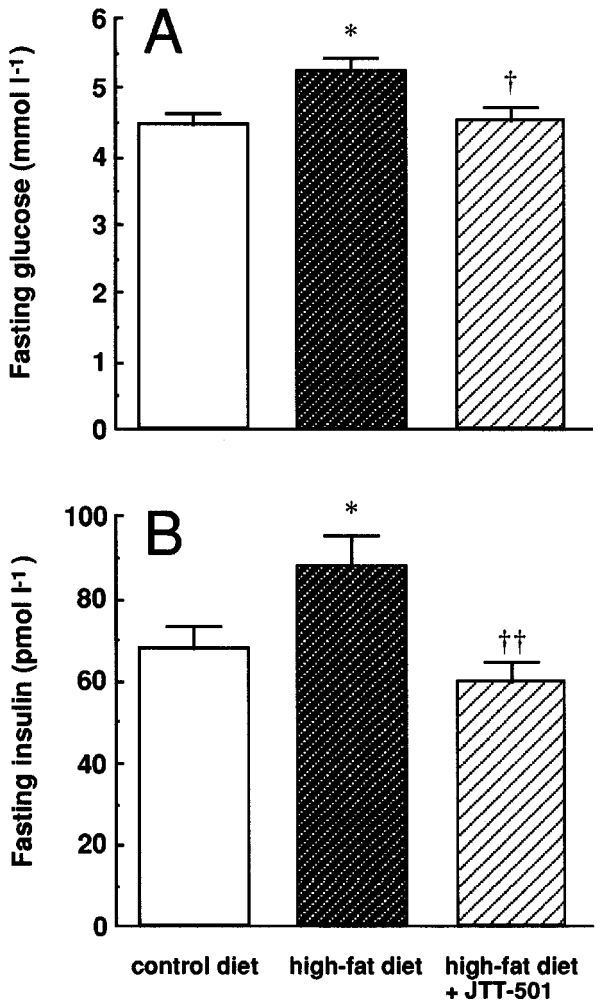

Figure 2 Effects of JTT-501 on fasting serum glucose and insulin concentrations in high-fat fed rats. JTT-501 (100 mg kg^{-1} day^{-1}) was orally administered to high-fat fed rats for the last 7 days of the diet period. Rats were fasted for 16 h after the last administration, blood samples were taken from the tail vein. Each column represents the means±s.e.mean ($n=8$). *$P<0.05$, **$P<0.01$ vs control rats, †$P<0.05$, ††$P<0.01$ vs high-fat fed rats.

lean rats (Table 1). JTT-501 tended to reduce both areas and decreased the IR index of Zucker fatty in a dose dependent manner. There was a significant difference in insulin resistance index between Zucker fatty rats and the JTT-501 100 mg kg^{-1} day^{-1} group.

Similar results were obtained in high-fat fed rats. The augmentation of glucose and insulin curve area, and IR index in high-fat fed rats was higher than that of control rats (Table 1). JTT-501 reduced insulin area but not glucose area, and improved the IR index of high-fat fed rats.

Effect of JTT-501 on insulin-stimulated glucose oxidation and lipid synthesis

Basal glucose oxidation levels in adipose tissue of Zucker fatty rats were similar to those of lean rats, although insulin-stimulated glucose oxidation levels of Zucker fatty rats were decreased compared with those of lean rats (Figure 3A). Treatment of JTT-501 increased basal glucose oxidation, and shifted the insulin-dose response curve leftward and augmented the maximum response in a dose dependent manner. EC_{50} values in insulin concentration for Zucker fatty rats, lean rats, JTT-501 10, 30 and 100 mg kg^{-1} day^{-1} were >100, <0.1, 5.26, 0.44 and 0.28 mU ml^{-1}, respectively. Similar results were also seen for total lipid synthesis (Figure 3B).

Effect of JTT-501 in the euglycaemic-hyperinsulinaemic clamp study

Serum insulin levels of Zucker fatty rats were higher than those of lean rats (Table 2). The insulin-stimulated glucose infusion rate (GIR) of Zucker fatty rats was lower than that of lean rats. There was no significant difference in glucose concentration between Zucker fatty rats and lean rats. JTT-501 (100 mg kg^{-1} day^{-1}) decreased serum insulin levels and improved the impaired GIR of Zucker fatty rats.

Similar results were observed in high-fat fed rats. The insulin-stimulated GIR of high-fat fed rats was lower than that of control rats. JTT-501 (100 mg kg^{-1} day^{-1}) ameliorated the impaired GIR of high-fat fed rats.

Effect on autophosphorylation of the insulin receptor

Basal autophosphorylation levels of the insulin receptor β-subunits of hindlimb muscles of Zucker fatty rats and Zucker fatty rats treated with JTT-501 (100 mg kg^{-1} day^{-1}) were not significantly different (Figure 4A and B). Insulin-stimulated

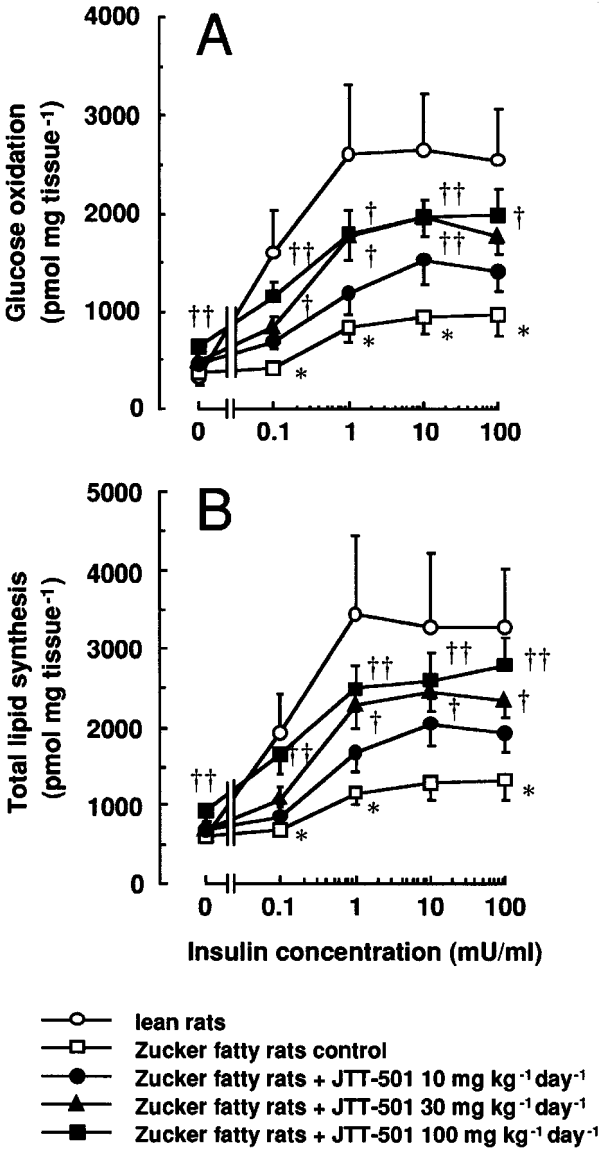

Figure 3 Effects of JTT-501 on insulin-stimulated glucose oxidation and lipid synthesis in adipose tissues from Zucker fatty rats. JTT-501 (10, 30 and 100 mg kg^{-1} day^{-1}) was orally administered to Zucker fatty rats for 21 days. On day 22, epididymal fat pads were removed, and glucose oxidation (A) and lipid synthesis (B) were measured. Each point represents the means ± s.e.mean ($n=5$). *$P<0.05$ vs lean rats, †$P<0.05$, ††$P<0.01$ vs Zucker fatty rats control.

Table 1 Effects of JTT-501 on glucose tolerance test and insulin resistance (IR) index

	n	*Area* (cm^2) *Glucose*	*Insulin*	*IR index* (units × 10^3)
Lean rats	5	15.2 ± 0.2	86.9 ± 10.2	1.3 ± 0.2
Zucker fatty rats	5	27.1 ± 0.8**	2530.6 ± 193.7**	68.9 ± 6.9**
JTT-501 10 mg kg^{-1} day^{-1}	5	24.1 ± 3.0	2357.4 ± 363.7	54.2 ± 7.3
JTT-501 30 mg kg^{-1} day^{-1}	5	24.5 ± 2.7	1765.6 ± 289.1	43.1 ± 8.0
JTT-501 100 mg kg^{-1} day^{-1}	5	20.9 ± 1.5	1397.8 ± 300.8	29.4 ± 6.9††
Control rats	8	17.4 ± 0.5	246.6 ± 17.1	4.3 ± 0.2
High-fat fed rats	8	20.4 ± 0.6¶¶	490.8 ± 44.2¶¶	10.1 ± 1.1¶¶
JTT-501 100 mg kg^{-1} day^{-1}	8	20.9 ± 0.3¶¶	348.2 ± 29.4¶¶§§	7.2 ± 0.6¶§

Data are expressed as the means ± s.e.mean. Values for glucose are given in mmol l^{-1} and for insulin in pmol l^{-1}. Area of the glucose and insulin curves was calculated by multiplying cumulative mean height of the glucose values (1 mmol l^{-1} = 1 cm) and insulin values (1 pmol l^{-1} = 1 cm), respectively, by time (60 min = 1 cm). IR index was calculated as the production of glucose and insulin area for each animal. **$P<0.01$ vs lean rats. ††$P<0.01$ vs Zucker fatty rats. ¶$P<0.05$, ¶¶$P<0.01$ vs control rats. §$P<0.05$, §§$P<0.01$ vs high-fat fed rats.

autophosphorylation levels of the insulin receptor β-subunits of Zucker fatty rats were lower than those of lean rats. JTT-501 significantly improved the impaired insulin-stimulated autophosphorylation levels of Zucker fatty rats.

Effect of JTT-501 on hyperleptinaemia

Zucker fatty rats showed remarkable obesity and hyperleptinaemia compared with lean rats (Tables 3 and 4). JTT-501 (100 mg kg^{-1} day^{-1}) reduced serum leptin concentrations of Zucker fatty rats in both the fed and fasted state, but did not affect body weight or epididymal fat pad weight.

On the other hand, high-fat fed rats showed visceral obesity (higher epididymal fat weight) and hyperleptinaemia compared with control rats, although body weights of high-fat fed rats were similar to those of control rats (Tables 3 and 4). JTT-501 decreased serum leptin concentrations in the fasted state, but did not affect body weight or fat weight.

Discussion

In the present study, we have demonstrated that JTT-501 improves the metabolic abnormalities of Zucker fatty rats and high-fat fed rats by enhancing insulin action in peripheral tissues.

Insulin resistance, characterized by reduced responsiveness to circulating insulin, is a common feature in almost all patients with NIDDM. Zucker fatty rats are generally used as an insulin-resistant model. In our study, Zucker fatty rats were hyperglycaemic, hyperinsulinaemic, hypertriglycedaemic and hyperleptinaemic, which is in keeping with previous reports (Fujiwara *et al*., 1988; Ghorbani & Himms-Hagen, 1998). JTT-501 decreased serum triglyceride and leptin levels, and tended to reduce serum glucose and insulin concentrations. JTT-501 clearly improved the IR index in glucose tolerance test, and increased glucose infusion rate in the euglycaemic-hyperinsulinaemic clamp study. Furthermore, in an *ex vivo* assay, JTT-501 enhanced insulin-dependent glucose oxidation and lipid synthesis, and improved the impaired insulin-stimulated autophosphorylation levels of the insulin receptor. These results suggest that JTT-501 improves insulin resistance by

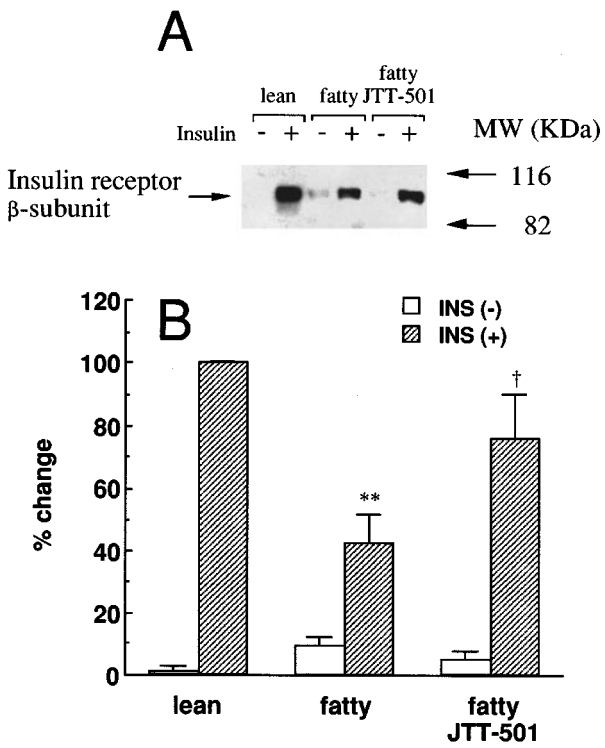

Figure 4 Effects of JTT-501 on insulin-stimulated autophosphorylation of the insulin receptor β-subunits in hindlimb muscles from Zucker fatty rats. JTT-501 (100 mg kg^{-1} day^{-1}) was orally administered to Zucker fatty rats for 7 days. Rats were fasted for 16 h after the last administration. The hindlimb muscles were removed 5 min after intravenous insulin (5 U kg^{-1}) or saline injection. The data are expressed as the immunoblotting data (A) and the densitometory data (B). Each column represents the mean±s.e.mean ($n=4$). $^{**}P<0.01$ vs lean rats, †$P<0.05$ vs Zucker fatty rats.

Table 2 Effect of JTT-501 on euglycaemic-hyperinsulinaemic clamp study

	n	*Glucose* (mmol l^{-1})	*Insulin* (pmol l^{-1})	*GIR* (mg kg^{-1} min^{-1})
Lean rats	7	8.5±0.4	2400±244	33.3±1.3
Zucker fatty rats	7	8.7±0.3	5756±444*	8.9±1.3*
JTT-501 treatment	7	9.1±0.2	3422±514†	21.1±1.0*†
Control rats	6	5.7±0.2	754±72	36.4±2.1
High-fat fed rats	6	5.8±0.2	1017±190	15.7±0.8¶
JTT-501 treatment	6	6.3±0.2	873±117	26.2±1.1¶§

Data are expressed as the means±s.e.mean. *$P<0.01$ vs lean rats. †$P<0.01$ vs Zucker fatty rats. ¶$P<0.01$ vs control rats. §$P<0.01$ vs high-fat fed rats.

Table 3 Effects of JTT-501 on body weight and epididymal fat weight

	n	*Body weight* (g) *Initial*	*Final*	*Fat weight* (g)
Lean rats	8	300.5±6.5	310.3±6.4	4.3±0.2
Zucker fatty rats	8	465.9±11.7*	499.4±10.6*	13.3±1.0*
JTT-501 treatment	8	466.5±8.0*	510.1±9.6*	14.7±0.6*
Control rats	10	246.6±7.6	274.0±7.6	2.7±0.1
High-fat fed rats	10	250.6±8.5	293.0±9.5	4.5±0.3¶
JTT-501 treatment	10	252.3±8.7	302.3±9.1	5.0±0.4¶

Data are expressed as the means±s.e.mean. *$P<0.01$ vs lean rats. ¶$P<0.01$ vs control rats.

Table 4 Effect of JTT-501 on serum leptin concentrations

	n	*Serum leptin* (ng ml^{-1}) *Fed*	*Fasted*
Lean rats	8	8.8±1.4	ND
Zucker fatty rats	8	699.0±44.3*	458.4±26.9*
JTT-501 treatment	8	534.1±29.7*†	347.5±15.0*†
Control rats	8	16.0±1.1	2.7±0.5
High-fat fed rats	8	59.7±7.8¶	16.2±1.7¶
JTT-501 treatment	8	51.8±7.4¶	10.3±1.2¶§

Data are expressed as the means±s.e.mean. ND: not detectable. *$P<0.01$ vs lean rats. †$P<0.01$ vs Zucker fatty rats. ¶$P<0.01$ vs control rats. §$P<0.01$ vs high-fat fed rats.

enhancing insulin sensitivity in peripheral tissues, and thereby improves the metabolic abnormalities of Zucker fatty rats.

The high-fat rat model is an alternative insulin-resistant model. We observed hyperglycaemia, hyperinsulinaemia, hyperleptinaemia and insulin resistance during the glucose tolerance test and the euglycaemic-hyperinsulinaemic clamp study. These abnormalities were identical with those reported in the literature (Maegawa *et al*., 1986; Stevenson *et al*., 1996; Ghorbani & Himms-Hagen, 1998). In this model, JTT-501 clearly improved the impaired insulin resistance index in the glucose tolerance test, and decreased abnormal serum parameters. These results also indicate that JTT-501 improves insulin resistance.

Insulin resistance is caused by abnormalities of the insulin receptor and of post-receptor signalling in cases of NIDDM and obesity (Maegawa, 1991; Grasso, 1995; Goodyear, 1995; Kahn, 1994). In the present study, we clarified that Zucker fatty rats have a defect of insulin-stimulated autophosphorylation of the insulin receptor, and demonstrated that the defect is clearly improved by JTT-501. With regard to pioglitazone and troglitazone, it has been reported that they improve insulin receptor defect in both *in vivo* (Iwanishi & Kobayashi, 1993; Kobayashi *et al*., 1992) and *in vitro* assays (Maegawa *et al*., 1993, 1995; Kellerer *et al*., 1994). This strongly suggests that elevation of insulin sensitivity in the peripheral tissues by JTT-501 is mediated by improvement of insulin receptor defects. Furthermore, there are also reports which have demonstrated the effectiveness of JTT-501 on post-receptor sites in insulin-resistant models. Obata *et al*. (1997) showed that acute and chronic treatment with JTT-501 increases insulin-stimulated phosphorylation of insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI-3-kinase) activity in muscles from Zucker fatty rats. In addition, Terasaki *et al*. (1998) demonstrated that JTT-501 restores insulin-stimulated phosphorylation of IRS-1 and activation of PI 3-kinase and improves insulin-induced glucose transporter 4 translocation to the plasma membrane in adipose tissues from high-fat fed rats. These reports support the hypothesis that JTT-501 affects not only the insulin receptor site but also post-receptor sites, while it is evident that JTT-501 improves insulin signalling defects in NIDDM and obesity.

Leptin is the protein product of *ob*, an adipocyte-specific gene, and reflects appetite, energy balance, and obesity (Caro *et al*., 1996). Mutation of the *ob* gene of *ob*/*ob* mice and the missense mutation in the Ob-receptor of *db*/*db* mice and Zucker fatty rats have been described (Zhang *et al*., 1994; Takaya *et al*., 1996; Philips *et al*., 1996). These mutations have not yet been found in humans; however, it is considered that leptin resistance is present in obese patients, since *ob* mRNA and plasma leptin levels are elevated in such patients and since there is a positive relationship between body mass index and plasma leptin levels (Maffei *et al*., 1995; Lönnqvist *et al*., 1995; Hamilton *et al*., 1995). In addition, insulin resistance is associated with elevated plasma leptin in lean and obese subjects (Segal *et al*., 1996). In the present study, we demonstrated that high-fat-fed rats and Zucker fatty rats have hyperleptinaemia and that JTT-501 reduces leptin levels in these models without affecting body weight or fat pad weight. Taken together, the results suggest that JTT-501 is effective in lowering leptin concentrations without regard to Ob-receptor mutation and that its lowering effect may further reflect improvement of leptin resistance.

It is speculated that multiple environmental and genetic factors participate in the pathogenesis of insulin resistance (Taylor, 1989; O'Doherty *et al*., 1997). Therefore, our present data indicating the effectiveness of JTT-501 in both genetic and non-genetic obese/insulin-resistant models may lead in the future to the amelioration of the extensive insulin-resistant conditions in NIDDM and obesity patients by JTT-501.

In this report, we have demonstrated that JTT-501 improves the metabolic abnormalities in genetic and non-genetic insulin-resistant models by enhancing insulin action in peripheral tissues. The mechanism of action of JTT-501 is due, at least in part, to elevating insulin receptor autophosphorylation. In addition, JTT-501 improves hyperleptinaemia in these models. We expect the novel antidiabetic agent, JTT-501, to show promise for treating NIDDM patients with insulin resistance.

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