Mechanism of Amelioration of Insulin Resistance by β_3-Adrenoceptor Agonist AJ-9677 in the KK-A^y/Ta Diabetic Obese Mouse Model

Hiroshi Kato, Mayumi Ohue, Kaori Kato, Akinori Nomura, Kaoru Toyosawa, Yasuji Furutani, Satoshi Kimura, and Takashi Kadowaki

The mechanism by which the specific β_3-adrenoceptor agonist AJ-9677 relieves insulin resistance in vivo was investigated by studying its effects in the white and brown adipose tissues of the KK-A^y/Ta diabetic obese mouse model. AJ-9677 reduced the total weight of white adipose tissues by reducing the size of the adipocytes, an effect associated with the normalization of tumor necrosis factor-α (TNF-α) and leptin expression levels. The levels of uncoupling protein (UCP)-1 mRNA in brown adipose tissue were increased threefold. AJ-9677 caused a marked increase (20- to 80-fold) in the expression of UCP-1 in white adipose tissues. The levels of UCP-2 mRNA were increased in both the white and brown adipose tissues of diabetic obese mice, and AJ-9677 further upregulated UCP-2 mRNA levels in brown adipose tissue, but reduced its levels in white adipose tissue. UCP-3 mRNA levels were not essentially changed by AJ-9677. However, AJ-9677 significantly (two- to fourfold) upregulated the GLUT4 mRNA and protein levels in white and brown adipose tissues and the gastrocnemius. The generation of small adipocytes, presumably mediated by increased expression of UCP-1 in addition to increased lipolysis in response to AJ-9677, was associated with decreased TNF-α and free fatty acid production and may be the mechanism of amelioration of insulin resistance in KK-A^y/Ta diabetic obese mice. *Diabetes* 50:113–122, 2001

The β_3-adrenoceptor was suspected to exist more than 15 years ago (1), and it is now known to be present in both rodent and human tissues. The human β_3-adrenoceptor was cloned and sequenced in 1989 (2). The characteristics of the β_3-adrenoceptor are quite different from those of β_1- and β_2-adrenoceptors. The β_3-adrenoceptor is expressed mainly in the white and brown adipose tissues (3), and it is important in lipolysis and thermogenesis in rodents (4). The β_3-adrenoceptor agonist BRL 26830A stimulates lipolysis in adipose tissues, and chronic treatment with an adequate dose of BRL 26830A has decreased body weight without reducing food intake (5). This weight loss was caused by the stimulation of energy expenditure and increased lipolysis (6). The effects of the β_3-adrenoceptor on thermogenesis are believed to occur through activation and upregulation of uncoupling protein (UCP)-1, which is mainly expressed in brown adipose tissues. For example, chronic treatment of yellow KK mice with the β_3-adrenoceptor agonist CL 316,243 increased expression of UCP-1 mRNA in brown adipose tissues and induced UCP-1 mRNA in white adipose tissues, the gastrocnemius, and quadriceps muscles (7). Recently, UCP-2 and -3 were identified. UCP-2 is widely expressed in humans; UCP-2 mRNA has been detected in the adipose tissues, skeletal muscles, lungs, the heart, and kidneys (8,9). UCP-3 is expressed primarily in skeletal muscles in humans and in skeletal muscles and brown adipose tissues in rodents (10,11). Regulation of the activation of UCP-2 and -3 may be different from that of UCP-1 (10,12,13), and these two proteins may be functionally involved in energy expenditure (8,10). Therefore, the increased energy expenditure induced by β_3-adrenoceptor agonists in vivo may be mediated by UCP-2 and -3 in addition to UCP-1, especially in humans.

β_3-adrenoceptor agonists have antidiabetic and antiobesity effects in rodent models of type 2 diabetes (14–17), but the molecular mechanisms of these effects, especially the antidiabetic effects, are largely unknown. Molecules secreted by hypertrophic adipocytes (e.g., tumor necrosis factor-α [TNF-α] and free fatty acids [FFAs]) may cause insulin resistance associated with obese type 2 diabetes. The expression levels of TNF-α in white adipose tissues are higher in diabetic obese models than in normal animals, and both neutralization of TNF-α (18) and target disruption of the TNF-α/TNF-α receptor (19) have prevented the development of insulin resistance despite the presence of obesity. TNF-α appears to interfere with the insulin signal transduction pathway by inhibiting the insulin receptor tyrosine kinase and the tyrosine phosphorylation of insulin receptor substrate-1 (18). Moreover, FFAs cause insulin resistance in the skeletal muscles and liver via multiple mechanisms, including the inhibition of phosphatidylinositol 3-kinase activity associated with insulin receptor substrate-1 (20), which may cause inhibition of GLUT4 translocation and glycogen synthesis stimulated by insulin. The expression levels of leptin in white adipose tissues and the plasma levels secreted from adipocytes are higher in diabetic obese models. Administration of leptin causes insulin sensitivity rather than insulin resistance (21). Leptin may be secreted from hypertrophic

From the Department of Pharmacology III (H.K., M.O., K.K., Y.F.), Discovery Research Laboratories; the Research and Development Coordination (A.N.) and the Department of Toxicology (K.T.), Developmental Research Laboratories, Dainippon Pharmaceutical Co., Osaka; and the Department of Metabolic Diseases (H.K., S.K., T.K.), Graduate School of Medicine, University of Tokyo, Tokyo, Japan.

Address correspondence and reprint requests to Hiroshi Kato, Department of Pharmacology III, Discovery Research Laboratories, Dainippon Pharmaceutical Co., Ltd., Osaka 564-0053, Japan. E-mail: hiroshi-kato@dainippon-pharm.co.jp.

ELISA, enzyme-linked immunosorbent assay; FFA, free fatty acid; NEFA, nonesterified fatty acid; OGTT, oral glucose tolerance test; PCR, polymerase chain reaction; PPAR-γ, peroxisome proliferator–activated receptor γ; PVDF, polyvinylidene difluoride; RT, reverse transcription; TNF-α, tumor necrosis factor-α; UCP, uncoupling protein.

adipocytes to compensate for the insulin resistance caused by obesity. In fact, some methods used to cause insulin resistance (e.g., lipid infusion and glucose infusion) were recently found to stimulate leptin gene expression (22). Thiazolidinediones improve insulin sensitivity in diabetic obese models. They increase the number of small white adipocytes and decrease the number of large adipocytes, thereby reducing expression levels of TNF-α and plasma FFA concentrations (23). The regulation of TNF-α expression and FFA production in white adipose tissues may be closely involved in the antidiabetic effect of β_3-adrenoceptor agonists.

Many β_3-adrenoceptor agonists have been developed, but the early compounds did not show the expected effects in humans that were seen in mice and rats. After the cloning of the human β_3-adrenoceptor, differences between the structures of the rat and human receptors were clarified. AJ-9677 was screened with Chinese hamster ovary cells expressing either the human or the rat β_3-adrenoceptor (24). We previously demonstrated that AJ-9677 could stimulate both rat and human β_3-adrenoceptors (24). The present study investigated the effects on diabetes and obesity and the molecular mechanisms of AJ-9677 in the KK-A^y/Ta diabetic obese mouse model.

RESEARCH DESIGN AND METHODS

Chemicals. AJ-9677 ([3-[(2*R*)-[[(2*R*)-(3-chlorophenyl)-2-hydroxyethyl]amino] propyl]-1*H*-indol-7-yloxy]acetic acid), a specific β_3-adrenoceptor agonist (β_1:β_2:β_3 = 1:2:210), was synthesized at Dainippon Pharmaceutical (Osaka, Japan).

Animals and drug treatment. Male 9-week-old KK-A^y/Ta and 11-week-old C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan) and were allowed to adjust to the facilities and handling for 1 week before all experimental protocols. Under standardized conditions, the mice were given dry food (CE-2; Oriental Yeast, Tokyo) and water ad libitum. Mice were divided into control and treatment groups so that the mean body weight of the two groups was comparable 1 day before the starting day (day 1). The treatment group was given AJ-9677 by gavage at 0.01 mg/kg or 0.1 $mg^{-1} \cdot kg^{-1} \cdot day^{-1}$ in the morning for 14 days. The control group was given only 0.5% tragacanth solution. At 24 h after the last administration of AJ-9677, blood samples were collected from the cut ends of the tails and centrifuged to separate plasma. Blood samples from 12-week-old C57BL/6J mice were also collected by the same method. For some mice, the rectal temperature was measured after AJ-9677 administration. Some mice were killed by bleeding under anesthesia 20 h after the final administration of AJ-9677. Epididymal white adipose tissues, inguinal white adipose tissues, interscapular brown adipose tissues, and the gastrocnemius were removed and divided into segments for further studies. The same tissues from 12-week-old C57BL/6J mice were also removed. Some KK-A^y/Ta mice were administered AJ-9677 (0.1 mg/kg) after 24-h fasting to observe the acute effect on lipolytic activity in vivo. Blood samples were collected 1 h after the administration.

Oral glucose tolerance test and determination of plasma glucose, insulin, FFAs, and triglycerides. The oral glucose tolerance test (OGTT) was performed on day 15 after 24 h fasting. The mice received a 20% glucose solution (2 g/kg). Blood samples were collected just before and 0.5, 1, 2, and 3 h after glucose loading. Plasma glucose and insulin levels were determined with a Glucose C-II Test from Wako Pure Chemical Industries (Osaka, Japan) and an insulin enzyme-linked immunosorbent assay (ELISA) from Shibayagi, (Gumma, Japan). FFAs were determined with a nonesterified fatty acid (NEFA) C-Test from Wako Pure Chemical Industries, and Triglycerides were detected with a Determiner triglyceride-S555 from Kyowa Medex Co. (Tokyo, Japan).

Lipolytic activity in white adipocytes. White adipocytes were isolated from epididymal white adipose tissues of KK-A^y/Ta mice by collagenase type II (Sigma Chemical, St. Louis, MO) digestion according to the method described by Rodbell (25). Adipocytes were successively filtered through 1,000-, 500-, and 200-μm meshes and washed in Krebs-Ringer phosphate solution (pH 7.4) containing bovine serum albumin (4 g/100 ml) and glucose (1 mg/ml). Isolated adipocytes were suspended in Krebs-Ringer phosphate solution (pH 7.4) containing bovine serum albumin (1 g/100 ml), glucose (1 mg/ml), phenylisopropyl adenosine (100 nmol/l), and adenosine deaminase (1 U/ml). Adipocytes (~2.5×10^5 cells/ml) were incubated with AJ-9677 or (–)-isoproterenol for 30 min at 37°C. The reaction was terminated with 1N H_2SO_4 and neutralized with 1N NaOH. FFAs were determined with an NEFA C-Test (Wako Pure Chemical Industries). A quantity of the isolated adipocytes was mixed in chloroform-methanol (3:2), shaken vigorously, and centrifuged at 10,000*g*. The precipitate was dissolved in 1N NaOH, and protein was determined with a bicinchoninic acid protein assay reagent (Pierce, IL).

Expression of TNF-α and leptin. Measurements of TNF-α and leptin proteins secreted in isolated white adipose tissues (400–1,000 mg) were performed as described previously (26). Adipose tissues were minced into small pieces and incubated in a Krebs-Ringer phosphate solution (pH 7.4) containing endotoxin-free bovine serum albumin (4 g/100 ml) and glucose (1 mg/ml) at 37°C with rotation (100 rpm). After a 2-h incubation, the incubation mixture was centrifuged and the supernatant collected. Concentrations of TNF-α and leptin proteins were determined with a mouse TNF-α ELISA system (Amersham Pharmacia Biotech U.K., Buckinghamshire, U.K.) and a mouse leptin ELISA kit (Morinaga Bioscience Institute, Yokohama, Japan). Total DNA was also isolated from the adipose tissues as described previously (27).

Histological analysis. Small pieces of epididymal and inguinal white adipose tissues were removed and rinsed with saline. The tissues were fixed with 10% formalin and embedded in paraffin. Tissue sections were cut at a thickness of 2.5 μm and stained with hematoxylin and eosin. To examine the size of the white adipocytes, the number of adipocytes was counted in five limited areas (7×10^{-3} mm^2) of each stained specimen. The mean value of the five areas was designated as an index of the cell size (i.e., a larger number means smaller size). In this analysis, the multilocular adipocytes were excluded. For immunohistochemistry, the paraffin-embedded sections were stained with anti–cytochrome oxidase antibody (Molecular Probes, Eugene, OR). The signals were detected by a Vectastain avitin-biotin-peroxidase complex system with a diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA). For electron microscopic observations, the tissues were fixed with 2.5% glutaraldehyde and embedded in Epon 812.

Triglycerides and DNA in adipose tissues. The method for determining the triglyceride and DNA contents in the epididymal and inguinal white adipose tissues was slightly modified from the method described by Okuno et al. (23). Briefly, 50 mg adipose tissue was homogenized in 2 ml of a solution containing 150 mmol/l sodium chloride, 0.1% Triton X-100, and 10 mmol/l Tris, pH 8.0, at 50°C using a polytron homogenizer (NS-310E; Micro Tech Nichion, Chiba, Japan) for 1 min. The triglyceride content of this homogenized solution was determined by a Determiner triglyceride-S555 (Kyowa Medex). For DNA determination, the homogenized solution was mixed with SDS, proteinase K, and EDTA to final concentrations of 0.1%, 100 μg/ml, and 10 mmol/l, respectively. After a 12–16 h incubation at 37°C, the DNA was extracted by the phenol-chloroform extraction method. The DNA pellets were redissolved in a solution containing 20 μg/ml ribonuclease A, 1 mmol/l EDTA, and 10 mmol/l Tris at pH 8.0. After 20 min of incubation at 37°C, the DNA was re-extracted by the phenol-chloroform extraction method. The DNA pellets were finally dissolved with TE buffer (10 mmol/l Tris and 1 mmol/l EDTA, pH 8.0). DNA content was calculated from the absorbance at 260 nm, with the optical density at 260 nm of 50 μg/ml DNA solution taken as equal to 1.0 by a Gene Quant RNA/DNA Calculator (Amersham Pharmacia Biotech, U.K.).

Expression of mRNAs by reverse transcription–competitive polymerase chain reaction. Extraction of total RNA from tissues was carried out by homogenizing 50 mg frozen tissue in 1 ml RNAzol B (Tel-Test, Friendswood, TX). RNA pellets were obtained from the homogenate by repeated extraction with chloroform and alcohol precipitation. The mRNA of TNF-α, leptin, GLUT4, and three different subtypes of UCP were quantitatively determined by the reverse transcription (RT)–competitive polymerase chain reaction (PCR) method described by Auboeuf and Vidal (28); however, the internal standard was prepared according to the method of Celi et al. (29). Table 1 lists both the sequences of primers used for internal standards and RT-competitive PCR and the sizes of each target fragment and competitor fragment generated by RT-competitive PCR. For construction of single-strand cDNA, 1 μg of total RNA was used with the SuperScript preamplification system (Life Technologies, Gaithersburg, MD) using oligo$(dT)_{12–18}$ as a primer. The internal standards (competitors) were prepared by PCR using template cDNA constructed with total RNA from epididymal white or interscapular brown adipose tissues and a specific set of oligonucleotides as primers (i.e., forward-short [S] and reverse) (Table 1). RT-competitive PCR was performed with a constant amount of target cDNA and four different amounts of the corresponding internal standard. The PCR conditions were 20–30 cycles of denaturation at 94°C for 15 s, annealing at 55°C (or 62°C for TNF-α) for 30 s, and extension at 72°C for 30 s. The number of cycles was selected to obtain a measurable amount of products depending on the content of the target. The PCR products were subjected to agarose gel electrophoresis and ethidium bromide staining. After photographs were taken, the density of the DNA band was analyzed with the Kodak Digital Science Electrophoresis Documentation and Analysis System 120 (Eastman Kodak Company, Rochester, NY). The initial amount of the target was calculated from the plot of the density ratio of the competitor band to the target band versus the initial amount of the competitor added in the PCR reaction.

Western blot analysis of GLUT4. Crude and plasma membranes were prepared from frozen epididymal white adipose tissues using differential ultracentrifugation as described by Simpson et al. (36) and Kelada et al. (37). Proteins

TABLE 1
Primers used for RT-competitive PCR

Target molecules	Sequences for primers	Locations	Product sizes (bp)	References
UCP-1				
Forward-L	:5′ AAA GCT TGT CAA CAC TTT GG 3′	503–522,	Target : 418	(30)
Forward-S	:5′ AAA GCT TGT CAA CAC TTT GGT AAC ATA TGA CCT CAT G 3′	503–522, and 578–594	Competitor : 363	
Reverse	:5′ GTG GTA CAA TCC ACT GTC TG 3′	901–920		
UCP-2				
Forward-L	:5′ GAC CTA TGA CCT CAT CAA AGA 3′	585–605,	Target : 286	(9)
Forward-S	:5′ GAC CTA TGA CCT CAT CAA AGA CCC TTG CCA CTT CAC TTC TG 3′	585–605, and 642–661	Competitor : 250	
Reverse	:5′ GGT GAC AAA CAT CAC TAC G 3′	852–870		
UCP-3				
Forward-L	:5′ AAG CCA TGA TAC GCC TGG GAA 3′	425–445,	Target : 362	GenBank
Forward-S	:5′ AAG CCA TGA TAC GCC TGG GAA GGC CCA ACA TCA CAA GA 3′	425–445, and 536–552	Competitor : 272	AF032902
Reverse	:5′ CTG AGC CAC CAT CTT CAG CAT 3′	766–786		
Leptin				
Forward-L	:5′ GTT TTG GAG CAG TTT GGA TC 3′	2,528–2,547,	Target : 522	(32)
Forward-S	:5′ GTT TTG GAG CAG TTT GGA TCC AGG TCA TAC CCT GTG GAG 3′	2,528–2,547, and 2,652–2,670	Competitor : 418	
Reverse	:5′ GCA TAT GGG AAG TTT CAC AA 3′	3,030–3,049		
TNF-α				
Forward-L	:5′ GGG ACA GTG ACC TGG ACT GT 3′	891–910,	Target : 520	(33)
Forward-S	:5′ GGG ACA GTG ACC TGG ACT GTA GGT TGC CTC TGT CTC AGA A 3′	891–910, and 1,009–1,028	Competitor : 422	
Reverse	:5′ GCA GAG GTT CAG TGA TGT AG 3′	1,391–1,410		
GLUT4				
Forward-L	:5′ TAG AGC AGG AGG TGA AAC CC 3′	2,066–2,085,	Target : 363	(34)
Forward-S	:5′ TAG AGC AGG AGG TGA AAC CCT CCT TTC CTC TAC AGC A 3′	2,066–2,085, and 2,172–2,188	Competitor : 277	
Reverse	:5′ TGC AGA CCC CTT CTC GAA AG 3′	2,409–2,428		
β-actin (for noncoding region)				
Forward-L	:5′ GGT TGG AGC AAA CAT CCC CC 3′	1,351–1,370,	Target : 460	(35)
Forward-S	:5′ GGT TGG AGC AAA CAT CCC CCA AGT GGT TAC AGG AAG TCC C 3′	1,351–1,370, and 1,471–1,490	Competitor : 360	
Reverse	:5′ TTG TGT AAG GTA AGG TGT GC 3′	1,791–1,810		
β-actin (for coding region)				
Forward-L	:5′ CGT GGG CCG CCC TAG GCA CCA 3′	102–122,	Target : 541	(35)
Forward-S	:5′ CGT GGG CCG CCC TAG GCA CCA ACT GGG ACG ACA TGG AG 3′	102–122, and 233–249	Competitor : 431	
Reverse	:5′ CTC TTT GAT GTC ACG CAC GAT TTC 3′	619–642		

For RT-competitive PCR, forward-long (L) and reverse primers were used, except that the forward-S primer was used instead of the forward-L primer to prepare internal standard (competitor). To standardize the amount of the target molecule, the amount of β-actin mRNA was determined using either the noncoding region primer set or the coding region primer set (the latter being used only for the gastrocnemius muscle β-actin mRNA).

in crude and plasma membrane fractions were separated by SDS-PAGE on 12.5% gels using the system described by Laemmli (38). The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes at 10 V for 30 min by the semidry blotting system (Bio-Rad Laboratories, Hercules, CA). The PVDF membranes were blocked with Block Ace (Dainippon Pharmaceutical) and incubated for 1 h with polyclonal anti-GLUT4 antibody (Transformation Research, Framingham, MA). The membranes were subsequently incubated with anti–rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The immunolabeled signals were detected with autoradiography film using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Statistical analysis. Values are shown as the means ± SE. The statistical significance of the values was analyzed by the Dunnett's two-tailed test when more than three groups were compared or the two-tailed Student's t test or the Welch t test when two groups were compared.

RESULTS

Effects of AJ-9677 on plasma glucose, insulin, FFA, and triglyceride levels. Control KK-A^{y}/Ta diabetic obese mice showed marked hyperglycemia, hyperinsulinemia, hypertriglyceridemia, and high FFA levels compared with normal C57BL/6J mice (Table 2). AJ-9677 stimulated lipolysis in vitro dose-dependently with higher potency than isoproterenol (Fig. 1). Acute administration of AJ-9677 also stimulated lipolysis, and plasma FFA levels were increased (0.77 ± 0.05 to 1.63 ± 0.05 mEq/l, $P < 0.001$, $n = 8$). However, in mice treated with AJ-9677 (0.1 mg/kg) for 14 days, hyperglycemia and high FFA levels were completely normalized and

TABLE 2
Effects of AJ-9677 treatment for 14 days on the body weight, total food intake, plasma parameters, weight of epididymal white adipose tissues, and triglyceride contents of white adipose tissues in KK-A^y/Ta diabetic obese mice and C57BL/6J mice

	KK-A^y/Ta		
	Control	AJ-9677 (0.1 mg/kg)	C57BL/6J
Body weight (g) on day 1	38.1 ± 0.6	37.8 ± 1.1	—
Body weight (g) on day 15	40.2 ± 0.7	37.8 ± 0.8	—
Body weight increase (%)	5.28 ± 0.53	0.03 ± 1.54*	—
Total food intake (g)	87.0 ± 4.0	87.0 ± 2.9	—
Plasma glucose (mg/dl)	421.1 ± 28.6	208.7 ± 10.3†	160.9 ± 9.2
Plasma insulin (ng/ml)	87.78 ± 11.84	4.15 ± 0.72†	1.35 ± 0.26
Plasma FFA (mEq/l)	0.61 ± 0.03	0.18 ± 0.01†	0.32 ± 0.04
Plasma triglyceride (mg/dl)	601.6 ± 28.1	217.1 ± 10.0†	121.0 ± 3.7
Epididymal WAT (g)	1.72 ± 0.05	0.91 ± 0.04†	0.26 ± 0.03
Triglyceride content in epididymal WAT (mg/μg DNA)	5.67 ± 0.70	3.09 ± 0.30†	2.66 ± 0.22
Triglyceride content in inguinal WAT (mg/μg DNA)	6.09 ± 0.66	3.05 ± 0.33*	1.93 ± 0.18

Data are means ± SE; n = 6–8 in KK-A^y/Ta groups and 5 in the C57BL/6J group. Blood and tissue samples were collected under well-fed conditions. *P < 0.01 and †P < 0.001 vs. the control group. WAT, white adipose tissue.

hyperinsulinemia and hypertriglyceridemia were markedly improved (Table 2). The OGTT indicated that the diabetic obese mice treated with AJ-9677 showed significantly lower glucose levels after glucose loading and significantly lower levels of plasma insulin than the untreated control mice (Fig. 2). These data clearly indicate that the AJ-9677 antidiabetic effect is mediated by the amelioration of insulin resistance in the diabetic obese mouse model. Although AJ-9677 treatment did not alter the total food intake, age-dependent weight gain was reduced in the diabetic obese mice (Table 2). These results suggest that AJ-9677 may inhibit weight gain by increasing energy expenditure. Consistent with this idea, rectal temperature was elevated by ~1°C (36.8 ± 0.2 to 37.7 ± 0.1°C, P < 0.01, n = 8) 1.5 h after the administration on day 1 of 0.1 mg/kg of AJ-9677.

Effects of AJ-9677 on white adipose tissues. The mice treated with AJ-9677 showed an ~50% reduction in the weight of epididymal white adipose tissues compared with the control mice (Table 2). Moreover, triglyceride levels in the

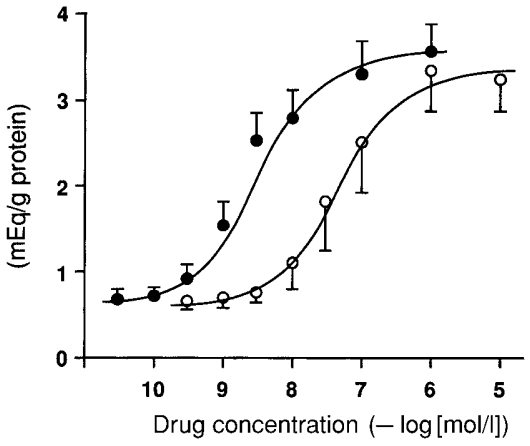

FIG. 1. Lipolytic activities of AJ-9677 in white adipocytes from KK-A^y/Ta diabetic obese mice. Isolated adipocytes were incubated with AJ-9677 (●) or isoproterenol (○). FFA concentrations were normalized to protein concentrations. The data are expressed as means ± SE of four experiments.

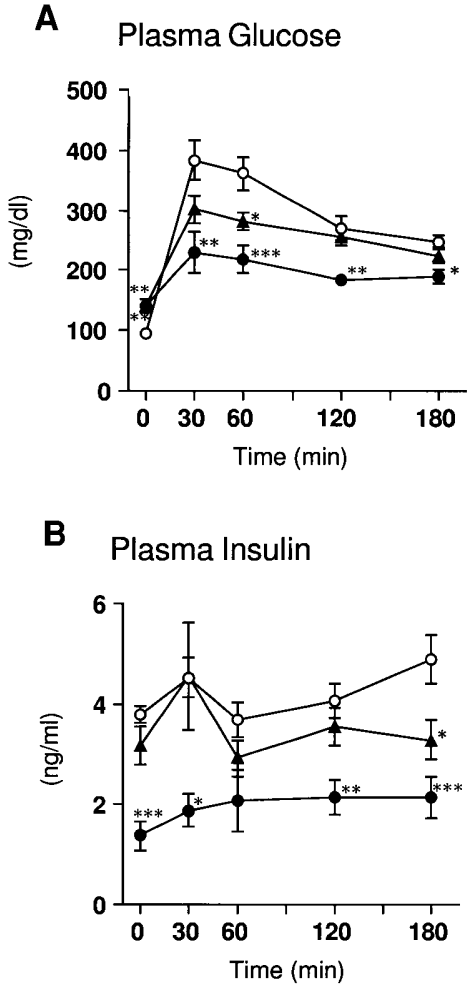

FIG. 2. Effects of AJ-9677 on glucose tolerance in KK-A^y/Ta diabetic obese mice. OGTT was performed after 24-h fasting after administration of AJ-9677 (0.01 mg^{-1} · kg^{-1} · day^{-1} or 0.1 mg^{-1} · kg^{-1} · day^{-1}) for 14 days. Blood samples were collected sequentially and plasma glucose (*A*) and insulin (*B*) were measured. ○, control; ▲, AJ-9677 (0.01 mg/kg); ●, AJ-9677 (0.1 mg/kg). The data are expressed as means ± SE; n = 6 in each group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the control group.

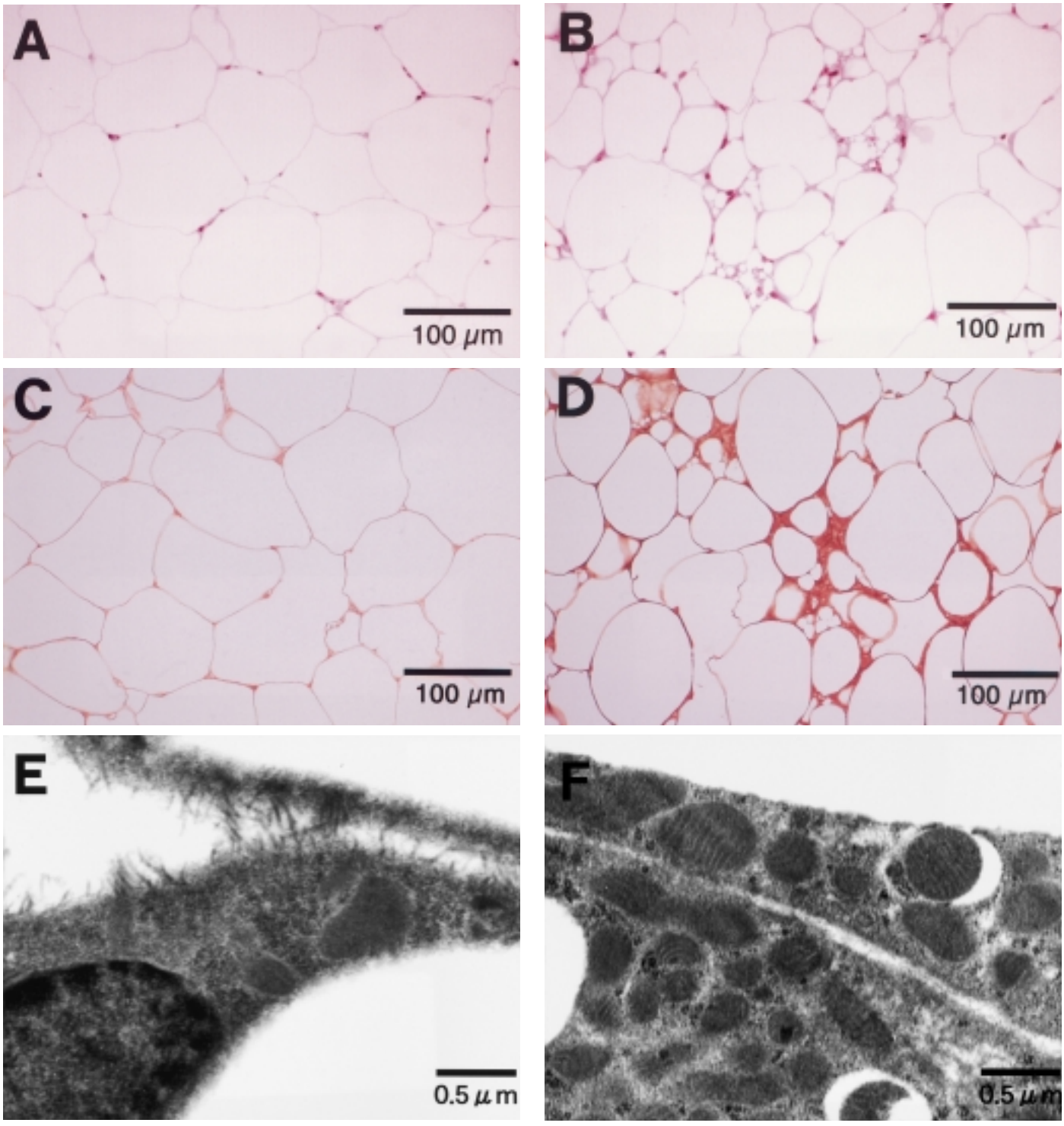

FIG. 3. Histological examination of white adipose tissues from control and AJ-9677–treated KK-A^y/Ta diabetic obese mice. Tissues were removed after a 14-day treatment under well-fed conditions. *A*, *C*, and *E* show control KK-A^y/Ta mice. *B*, *D*, and *F* show AJ-9677–treated KK-A^y/Ta mice. *A* and *B* show epididymal white adipose tissues stained with hematoxylin and eosin. *C* and *D* show epididymal white adipose tissues immunostained with anti-cytochrome oxidase antibody. *E* and *F* show electron microscopic observations of inguinal white adipose tissues.

epididymal white adipose tissues normalized to DNA levels (TG/DNA ratios) were also reduced by ~50%, to almost the same level as the control levels (Table 2), indicating that the triglyceride content per cell was reduced to normal. TG/DNA ratios in inguinal white adipose tissues were also reduced by ~50% by AJ-9677 treatment (Table 2).

Histological analysis of white adipose tissues. Treatment with AJ-9677 clearly reduced the size of adipocytes in the epididymal white adipose tissues (Fig. 3*A* and *B*). The number of white adipocytes in the limited area was larger in the AJ-9677–treated group than that in the control group. This effect was exerted in a dose-dependent manner (i.e., a

TABLE 3
Effects of AJ-9677 administration on the size of white adipocytes

	Number of cells (cells per 7×10^{-3} mm^2)
Experiment 1	
Control	91.7 ± 3.9
AJ-9677 (0.01 mg/kg)	111.6 ± 7.8*
Experiment 2	
Control	75.2 ± 3.1
AJ-9677 (0.1 mg/kg)	120.1 ± 2.3†

Data are means ± SE; n = 10 in each group.The number of cells were counted in five limited areas of each section stained with hematoxylin and eosin. *P < 0.05 and †P < 0.001 vs. the control group.

120% increase at a dosage of 0.01 mg/kg and a 160% increase at a dosage of 0.1 mg/kg) (Table 3). Moreover, the AJ-9677 treatment caused the formation of some multilocular adipocytes resembling brown adipocytes. Immunostaining of cytochrome oxidase in the AJ-9677–treated group was stronger than that in the control group (Fig. 3*C* and *D*). These results were also found in the inguinal white adipose tissues (data not shown). These observations suggest that AJ-9677 treatment caused the exaltation of mitochondrial function and the conversion of white adipocytes into brown adipocytes. Alternatively, β_3-adrenoceptor agonist treatment may cause differentiation of preadipocytes of brown adipose tissue lineage in white adipose tissues. AJ-9677 treatment caused a dramatic increase in the number of mitochondria in the cytoplasm of inguinal white adipocytes (Fig. 3*E* and *F*).

Effect of AJ-9677 treatment on the overexpression of TNF-α and leptin mRNA and protein. The TNF-α mRNA levels were markedly higher in control KK-A^y/Ta mice than in C57BL/6J mice. Treatment with AJ-9677 decreased the TNF-α mRNA level to 35% of the control level (Fig. 4*A*). This reduction of TNF-α mRNA was associated with a parallel reduction in the amount of TNF-α protein secreted from the adipocytes (Fig. 4*C*). Very similar data were obtained for leptin mRNA and protein secretion (Fig. 4*B* and *D*). AJ-9677 also reduced the mRNA expression levels and secretion of both TNF-α and leptin in the inguinal white adipose tissues (data not shown).

Effect of AJ-9677 treatment on expression of UCP-1, -2, and -3. AJ-9677 treatment caused the mRNA expression of UCP-1 in brown adipose tissues to be increased threefold (Fig. 5*A*). UCP-2 mRNA levels were increased in the brown adipose tissues of control KK-A^y/Ta mice, and AJ-9677 treatment further upregulated the expression of UCP-2 mRNA. UCP-3 mRNA levels were not changed in the brown adipose tissues of the control KK-A^y/Ta mice, and AJ-9677 treatment did not affect the expression of UCP-3 mRNA. UCP-1 was hardly expressed in the epididymal and inguinal white adipose tissues of both control KK-A^y/Ta and C57BL/6J mice. However, administration of AJ-9677 caused a 20- to 80-fold increase in UCP-1 expression levels in the epididymal (Fig. 5*B*) and inguinal (Fig. 5*C*) white adipose tissues. UCP-2 expression levels were increased three- to sixfold in both epididymal and inguinal white adipose tissues of the control KK-A^y/Ta mice, as seen in brown adipose tissues (Fig. 5*B* and *C*). However, the expression of UCP-2 mRNA was reduced and left unchanged by AJ-9677 in the epididymal and inguinal white adipose tissues, respectively, unlike the effects seen in brown adipose tissues (Fig. 5*B* and *C*). The expression of UCP-3 was not altered in white and brown adipose tissues of the control KK-A^y/Ta mice. Although AJ-9677 treatment decreased UCP-3 expression levels in epididymal white adipose tissues (Fig. 5*B*), it did not affect UCP-3 expression levels in

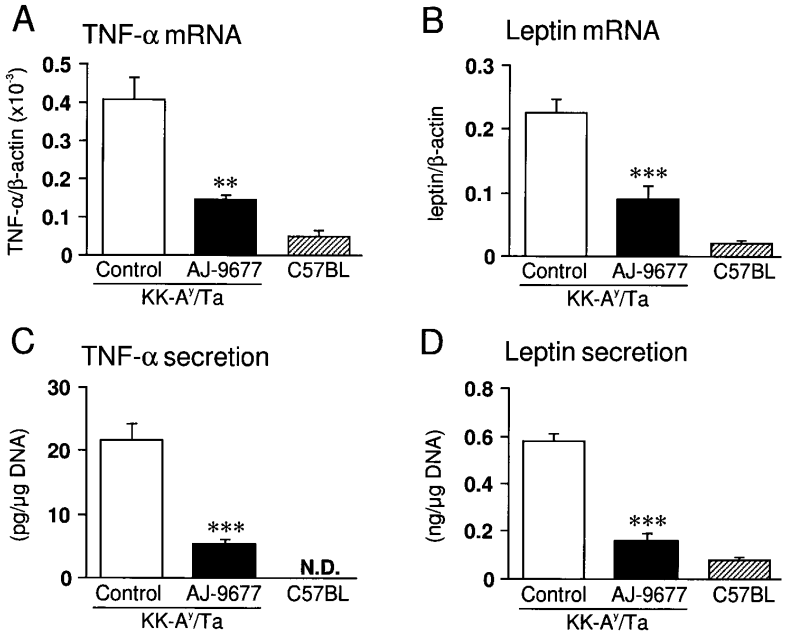

FIG. 4. Messenger RNA expression and secretion of TNF-α and leptin in epididymal white adipose tissues from control and AJ-9677–treated KK-A^y/Ta diabetic obese mice and C57BL/6J mice. Adipose tissues were removed after a 14-day treatment under well-fed conditions. *A* and *B*: TNF-α and leptin mRNA expression, respectively. The mRNA expression levels were analyzed by the RT-competitive PCR method. *C* and *D*: TNF-α and leptin protein secretion, respectively. TNF-α and leptin were measured by ELISA kits. The details of the experiments are described in RESEARCH DESIGN AND METHODS. The data are expressed as means ± SE; n = 8 in KK-A^y/Ta groups and n = 5 in the C57BL/6J group. **P < 0.01 and ***P < 0.001 compared with control KK-A^y/Ta group. N.D., not detected.

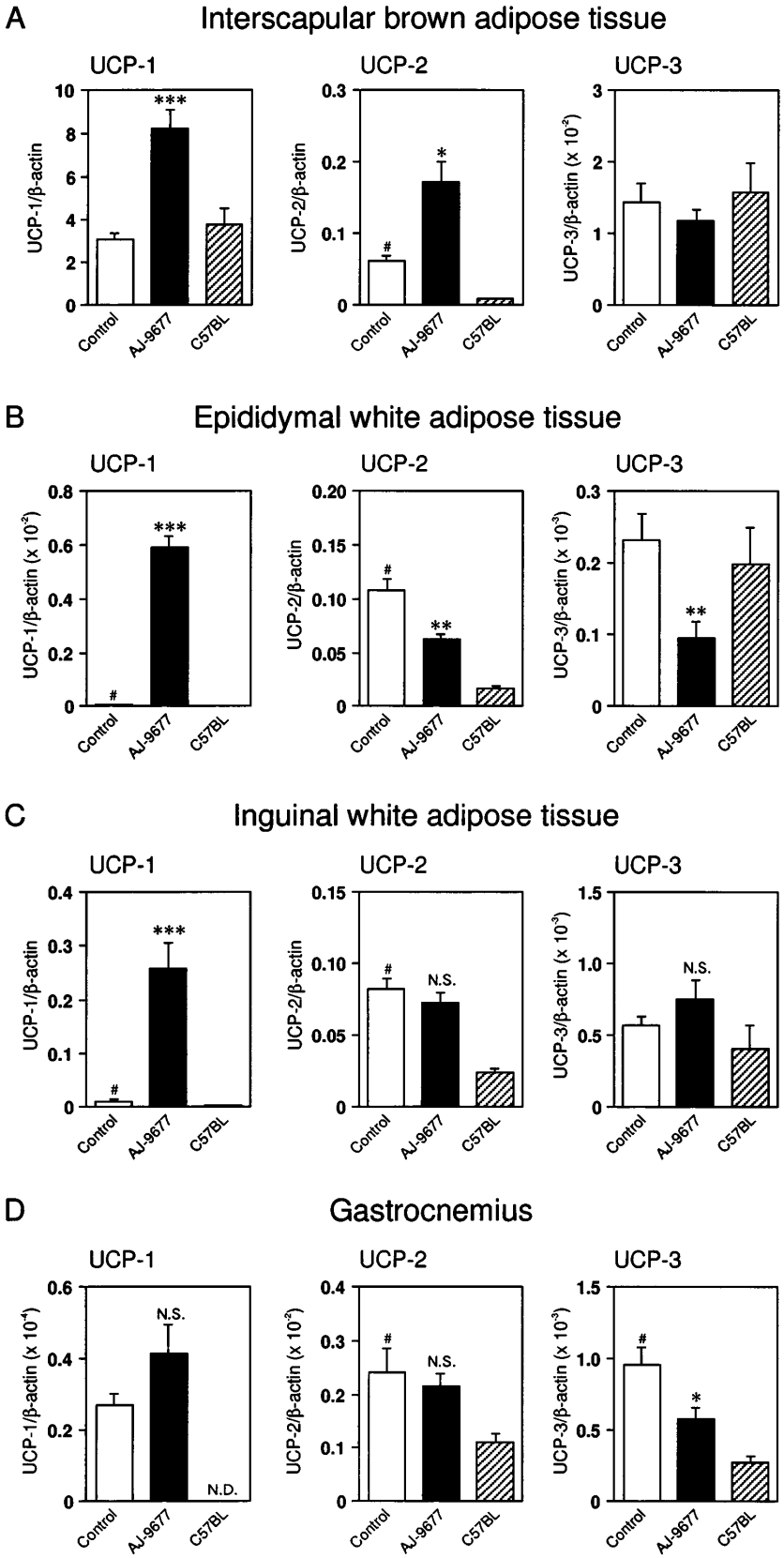

FIG. 5. Expression levels of UCP-1, -2, and -3 mRNA in adipose tissues and the gastrocnemius from control and AJ-9677–treated KK-A^y/Ta diabetic obese mice and C57BL/6J mice. Tissues were removed after a 14-day treatment under well-fed conditions. *A* shows interscapular brown adipose tissues; *B* shows epididymal white adipose tissues; *C* shows inguinal white adipose tissues; and *D* shows the gastrocnemius. The mRNA expression levels were analyzed by the RT-competitive PCR method. The details of the experiments are described in RESEARCH DESIGN AND METHODS. Data are expressed as means ± SE; n = 5–8 in KK-A^y/Ta groups and n = 5 in the C57BL/6J group. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the control KK-A^y/Ta group. N.S., not significant compared with the control KK-A^y/Ta group. #P < 0.05 compared with the C57BL/6J group.

TABLE 4
Effects of AJ-9677 treatment for 14 days on expression levels of GLUT4 mRNA in epididymal and inguinal white adipose tissues, interscapular brown adipose tissues, and the gastrocnemius in KK-A^y/Ta diabetic obese mice and C57BL/6J mice

	KK-A^y/Ta		
	Control	AJ-9677 (0.1 mg/kg)	C57BL/6J
Epididymal WAT	0.056 ± 0.003	0.207 ± 0.040†	0.059 ± 0.006
Inguinal WAT	0.281 ± 0.013	0.602 ± 0.081*	0.104 ± 0.029
Interscapular BAT	0.636 ± 0.151	1.672 ± 0.152‡	0.786 ± 0.114
Gastrocnemius	0.331 ± 0.056	0.826 ± 0.132†	0.609 ± 0.134

Data are means ± SE; n = 5–8 in each group. All values were normalized by the expression levels of β-actin mRNA. WAT, white adipose tissue; BAT, brown adipose tissue. *P < 0.05, †P < 0.01, and ‡P < 0.001 vs. the control group.

inguinal white adipose tissues (Fig. 5*C*). The expression levels of UCP-1, -2, and -3 mRNA in the gastrocnemius were very low compared with the adipose tissues, and they were not upregulated by AJ-9677 treatment (Fig. 5*D*).

Effect of AJ-9677 treatment on GLUT4 expression. GLUT4 mRNA expression in epididymal white adipose tissues was similar in control KK-A^y/Ta diabetic obese and C57BL/6J mice. AJ-9677 treatment increased GLUT4 mRNA expression approximately fourfold. AJ-9677 treatment also caused a two- to threefold increase in GLUT4 mRNA levels in inguinal white adipose tissues, brown adipose tissues, and the gastrocnemius (Table 4). AJ-9677 treatment also increased GLUT4 protein levels in the crude and plasma membrane of epididymal white adipose tissues four- and sixfold, respectively (Fig. 6).

DISCUSSION

Administration of AJ-9677 for 14 days reduced plasma glucose, insulin, FFA, and triglyceride levels in the diabetic obese mouse model to almost the normal levels seen in C57BL/6J mice. AJ-9677 stimulated lipolysis in adipocytes dose-dependently in vitro. Although the plasma FFA level increased after the first AJ-9677 administration, this effect was not observed after chronic treatment, presumably because plasma FFAs generated by lipolysis may be consumed by thermogenesis. Although we used the dosage of 0.1 $mg^{-1} \cdot kg^{-1} \cdot day^{-1}$, AJ-9677 can reduce these plasma parameters at lower dosages (data not shown). The effective dosage was much lower for AJ-9677 than for other β_3-adrenoceptor agonists reported previously (14,39). White adipose tissues in humans show lower expression levels of β_3-adrenoceptor than in rodents. However, because AJ-9677 is an equally potent agonist against both human and rat β_3-adrenoceptors in the Chinese hamster ovary cell expression system, it may show the same kind of effects, to some extent, in humans as it does in rodents.

Mitochondrial UCPs, which cause respiration without ATP synthesis, are believed to be involved in the expenditure of excess energy. At least three UCP isoforms (UCP-1, -2, and -3) have been cloned. UCP-1 is primarily expressed in brown adipose tissues, and it is slightly expressed in white adipose tissues. In the present study, administration of AJ-9677 increased the expression of UCP-1 threefold in brown adipose tissues and 20- to 80-fold in epididymal and inguinal white adipose tissues. The expression of UCP-2 was increased by 2.5-fold in brown adipose tissues, but it was decreased in epididymal white adipose tissues and unaltered in inguinal white adipose tissues. The expression of UCP-3 was not changed in brown adipose tissues and inguinal white adipose tissues, but it was decreased in epididymal white adipose tissues. Previous reports showed that the β_3-adrenoceptor agonists BRL 35135 and CL 316,243 (40–42) increased the expression of UCP-1 in both brown and white adipose tissues. Our data are consistent with those results. The effect of AJ-9677 on the expression of UCP-2 in brown adipose tissues is different from that of BRL 35135 (40), but consistent with CL 316,243 (41). The effects on UCP-3 expression are inconsistent. Thus, the effects of β_3-adrenoceptor agonists on UCP-2 and UCP-3 expression may depend on the types of β_3-adrenoceptor agonists, the duration of the treatment, and the time of death. Although CL 316,243 increased UCP-1 mRNA in the gastrocnemius (7), AJ-9677 did not increase UCP-1, 2, or 3 mRNA in the tissues. These

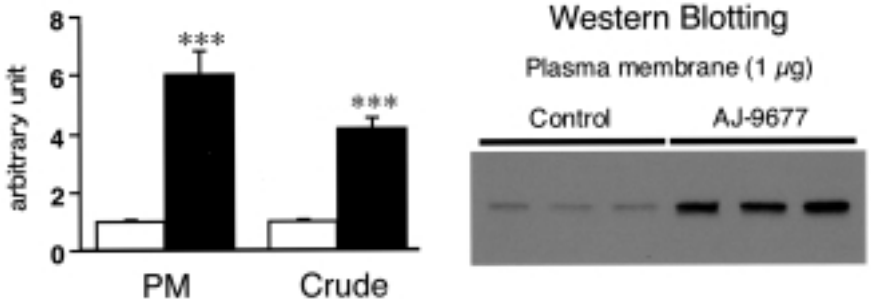

FIG. 6. Protein expression levels of GLUT4 in epididymal white adipose tissues from control and AJ-9677–treated KK-A^y/Ta diabetic obese mice. Tissues were removed after a 14-day treatment under well-fed conditions. Crude and plasma membrane fractions were prepared and analyzed by Western blotting method. The detail was described in RESEARCH DESIGN AND METHODS. Crude, crude membrane; PM, plasma membrane. □, control KK-A^y/Ta mice; ■, AJ-9677–treated KK-A^y/Ta mice. The data are expressed as means ± SE; n = 6 in each group. ***P < 0.001 compared with control KK-A^y/Ta group.

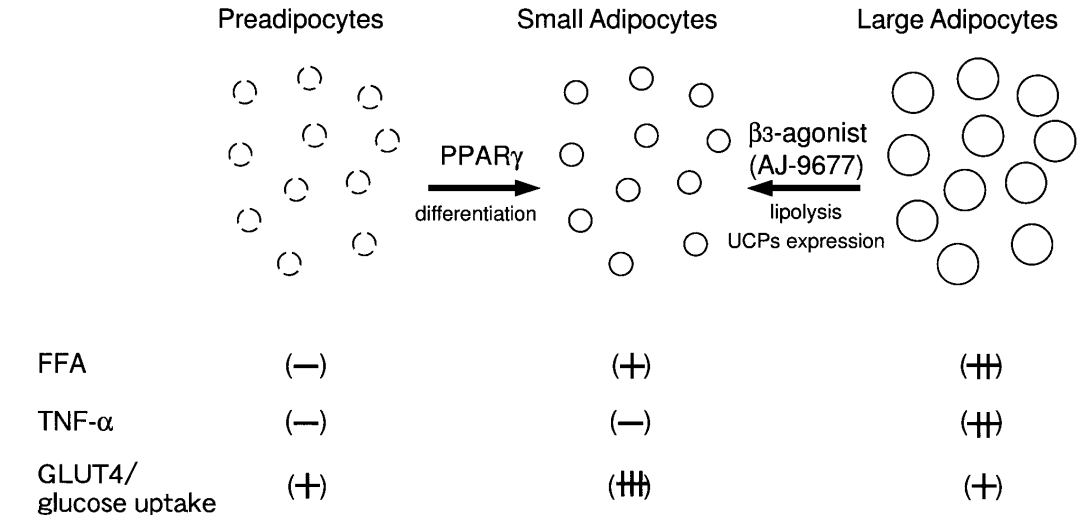

FIG. 7. Proposed mechanism of generation of small adipocytes and amelioration of insulin resistance by β_3-adrenoceptor agonist (AJ-9677). AJ-9677 converts large adipocytes into small adipocytes through increased lipolysis and the induction of uncoupling proteins (e.g., UCP-1) that are associated with the reduction of TNF-α and FFA, which leads to the amelioration of insulin resistance. Thiazolidinediones promote adipocyte differentiation to generate small adipocytes that are also associated with the reduction of TNF-α and FFA, which leads to the amelioration of insulin resistance. Therefore, insulin sensitivity is induced in vivo whether small adipocytes are generated de novo by differentiation via stimulation of PPAR-γ or by conversion from the large adipocytes via β_3-adrenergic receptors. The generation of small adipocytes is the key event in ameliorating insulin resistance.

differences may also depend on the types of β_3-adrenoceptor agonists. The role of UCPs in the muscles may not be very important for the antidiabetic effects of AJ-9677.

The major novel finding of this study is that the β_3-adrenoceptor agonist AJ-9677 reduced the size of white adipocytes, an effect associated with the reduction of mRNA expression and protein secretion of TNF-α and the reduction of plasma FFA levels. The reduction in size of the white adipocytes was confirmed by histological analysis and the triglyceride/DNA ratio. This reduction may be caused by increased lipolysis and energy expenditure mediated by the increased expression of both UCP-1 and UCP-2 after AJ-9677 treatment. We cannot identify the source of UCP-1 overexpressed in white adipose tissues; it is possible that the increased UCP-1 was expressed in the multilocular adipocytes found in the white adipose tissues during AJ-9677 treatment. However, because the absolute expression level of UCP-1 in white adipose tissues was much lower than in brown adipose tissues, white adipose tissues may contribute less to energy expenditure than brown adipose tissues. Thiazolidinediones cause the differentiation of preadipocytes into adipocytes (through the peroxisome proliferator–activated receptor γ [PPAR-γ]) to generate small white adipocytes and, concomitantly, cause the apoptosis of large white adipocytes (23). This reduction of the mean size of adipocytes by thiazolidinediones is associated with the normalization of both increased levels of expression of TNF-α and increased production of FFAs in diabetic obese animal models. Because the increase in the proportion of small adipocytes by thiazolidinediones appears to contribute to the amelioration of insulin resistance via decreased TNF-α expression and decreased FFA production, the reduction in the size of adipocytes by AJ-9677 may contribute to the amelioration of insulin resistance via a similar reduction of TNF-α expression and FFA production. Moreover, the reduction in the size of adipocytes by AJ-9677 may also be associated with decreased levels of leptin expression and secretion in white adipose tissues, which may well reflect the abrogation of the need for compensation for insulin resistance.

TNF-α can cause downregulation of GLUT4 expression (43,44), so decreased TNF-α expression probably contributes to increased GLUT4 expression after AJ-9677 treatment. Moreover, GLUT4 expression on the plasma membrane was preferentially increased compared with the crude membrane, suggesting that AJ-9677 treatment stimulated GLUT4 translocation to the plasma membrane in white adipose tissues. Whether this effect is a direct action of AJ-9677 or secondary to its amelioration of high plasma FFA levels is unclear at present. In either case, the change in the localization of GLUT4 to the plasma membrane, in addition to the increased GLUT4 expression levels, may further contribute to the amelioration of insulin resistance and diabetes.

We would like to propose a novel hypothesis for the relationship between the generation of small adipocytes and the amelioration of insulin resistance, as depicted in Fig. 7. The present study clearly shows that the β_3-adrenoceptor agonist AJ-9677 converted large adipocytes into small adipocytes through increased lipolysis and the induction of UCPs (e.g., UCP-1) that are associated with the reduction of TNF-α and FFA levels and thus the amelioration of insulin resistance. We previously showed that thiazolidinediones promote adipocyte differentiation to generate small adipocytes, which is also associated with reduction of TNF-α and FFA levels, leading to the amelioration of insulin resistance (23). Therefore, insulin sensitivity is induced in vivo whether small adipocytes are generated de novo by differentiation via stimulation of PPAR-γ or by conversion from the large adipocytes via β_3-adrenoceptors. Thus, we would like to propose that the generation of small adipocytes is the key event in the amelioration of insulin resistance.

Hypertrophic obesity resulting from adipocyte hypertrophy, which develops with a high-fat diet and sedentary lifestyle, is closely linked to major health issues (e.g., diabetes, hypertension, hyperlipidemia, and cardiovascular diseases) in Western

countries and in Japan (45). Insulin resistance, which is usually associated with hypertrophic obesity, is believed to be the major mechanism causing these diseases. Thus, the treatment of hypertrophic adipocytes is one of the most important issues in medical science. Thiazolidinediones may not be the ideal agents because the number of adipocytes tends to increase and promote obesity, even though insulin resistance is ameliorated. This study clearly demonstrated that β_3-adrenoceptor agonists (e.g., AJ-9677) appear to convert hypertrophic adipocytes into small adipocytes, thereby ameliorating insulin resistance and obesity simultaneously.

REFERENCES

1. Arch JR, Ainsworth AT, Cawthorne MA, Piercy V, Sennitt MV, Thody VE, Wilson C, Wilson S: Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature* 309:163–165, 1984
2. Emorine LJ, Marullo S, Briend-Sutren MM, Patey G, Tate K, Delavier-Klutchko C, Strosberg AD: Molecular characterization of the human β_3-adrenergic receptor. *Science* 245:1118–1121, 1989
3. Granneman JG, Lahners KN, Chaudhry A: Molecular cloning and expression of the rat β_3-adrenergic receptor. *Mol Pharmacol* 40:895–899, 1991
4. Atgie C, D'Allaire F, Bukowiecki LJ: Role of β_1- and β_3-adrenoceptors in the regulation of lipolysis and thermogenesis in rat brown adipocytes. *Am J Physiol* 273:C1136–C1142, 1997
5. Arch JR, Ainsworth AT, Ellis RD, Piercy V, Thody VE, Thurlby PL, Wilson C, Wilson S, Young P: Treatment of obesity with thermogenic β-adrenoceptor agonists: studies on BRL 26830A in rodents. *Int J Obes* 8 (Suppl. 1):1–11, 1984
6. Strosberg AD, Pietri-Rouxel F: Function and regulation of the β_3-adrenoceptor (Review). *Trends Pharmacol Sci* 17:373–381, 1996
7. Nagase I, Yoshida T, Kumamoto K, Umekawa T, Sakane N, Nikami H, Kawada T, Saito M: Expression of uncoupling protein in skeletal muscle and white fat of obese mice treated with thermogenic β_3-adrenergic agonist. *J Clin Invest* 97:2898–2904, 1996
8. Fleury C, Neverova M, Collins S, Raimbault S, Champigny O, Levi-Meyrueis C, Bouillaud F, Seldin MF, Surwit RS, Ricquier D, Warden CH: Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nat Genet* 15:269–272, 1997
9. Gimeno RE, Dembski M, Weng X, Deng N, Shyjan AW, Gimeno CJ, Iris F, Ellis SJ, Woolf EA, Tartaglia LA: Cloning and characterization of an uncoupling protein homolog: a potential molecular mediator of human thermogenesis. *Diabetes* 46:900–906, 1997
10. Boss O, Samec S, Paoloni-Giacobino A, Rossier C, Dulloo A, Seydoux J, Muzzin P, Giacobino JP: Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. *FEBS Lett* 408:39–42, 1997
11. Vidal-Puig A, Solanes G, Grujic D, Flier JS, Lowell BB: UCP3: an uncoupling protein homologue expressed preferentially and abundantly in skeletal muscle and brown adipose tissue. *Biochem Biophys Res Commun* 235:79–82, 1997
12. Millet L, Vidal H, Andreelli F, Larrouy D, Riou JP, Ricquier D, Laville M, Langin D: Increased uncoupling protein-2 and -3 mRNA expression during fasting in obese and lean humans. *J Clin Invest* 100:2665–2670, 1997
13. Boss O, Samec S, Dulloo A, Seydoux J, Muzzin P, Giacobino JP: Tissue-dependent upregulation of rat uncoupling protein-2 expression in response to fasting or cold. *FEBS Lett* 412:111–114, 1997
14. Carroll MJ, Lister CA, Sennitt MV, Stewart-Long N, Cawthorne MA: Improved glycemic control in C57Bl/KsJ (*db/db*) mice after treatment with the thermogenic β-adrenoceptor agonist, BRL 26830. *Diabetes* 34:1198–1204, 1985
15. Cawthorne MA, Carroll MJ, Levy AL, Lister CA, Sennitt MV, Smith SA, Young P: Effects of novel β-adrenoceptor agonists on carbohydrate metabolism: relevance for the treatment of non-insulin-dependent diabetes. *Int J Obes* 8 (Suppl. 1):93–102, 1984
16. Largis EE, Burns MG, Muenkel HA, Dolan JA, Claus TH: Antidiabetic and antiobesity effects of a highly selective β_3-adrenoceptor agonist (CL 316,243). *Drug Dev Res* 32:69–76, 1994
17. Yoshida T, Sakane N, Wakabayashi Y, Umekawa T, Kondo M: Anti-obesity and anti-diabetic effects of CL 316,243, a highly specific β_3-adrenoceptor agonist, in yellow KK mice. *Life Sci* 54:491–498, 1994
18. Hotamisligil GS, Budavari A, Murray D, Spiegelman BM: Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes: central role of tumor necrosis factor-α. *J Clin Invest* 94:1543–1549, 1994
19. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS: Protection from obesity-induced insulin resistance in mice lacking TNF-α function. *Nature* 389:610–614, 1997
20. Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, Shulman GI: Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* 103:253–259, 1999
21. Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ: Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature* 389:374–377, 1997
22. Wang J, Liu R, Hawkins M, Barzilai N, Rossetti L: A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. *Nature* 393:684–688, 1998
23. Okuno A, Tamemoto H, Tobe K, Ueki K, Mori Y, Iwamoto K, Umesono K, Akanuma Y, Fujiwara T, Horikoshi H, Yazaki Y, Kadowaki T: Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. *J Clin Invest* 101:1354–1361, 1998
24. Kawashima H, Nomura A, Ohue M, Kato H, Kuwajima J, Furutani Y, Hosoki K, Karasawa T: A novel human β_3-adrenoceptor agonist AD-9677 (Abstract). *Diabetologia* 40 (Suppl. 1):A374, 1997
25. Rodbell, M: Metabolism of isolated fat cells. *J Biol Chem* 239:375–380, 1964
26. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-α in human obesity and insulin resistance. *J Clin Invest* 95:2409–2415, 1995
27. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1989
28. Auboeuf D, Vidal H: The use of the reverse transcription-competitive polymerase chain reaction to investigate the in vivo regulation of gene expression in small tissue samples. *Anal Biochem* 245:141–148, 1997
29. Celi FS, Zenilman ME, Shuldiner AR: A rapid and versatile method to synthesize internal standards for competitive PCR. *Nucleic Acids Res* 21:1047, 1993
30. Kozak LP, Britton JH, Kozak UC, Wells JM: The mitochondrial uncoupling protein gene: correlation of exon structure to transmembrane domains. *J Biol Chem* 263:12274–12277, 1988
32. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425–432, 1994
33. Fransen L, Muller R, Marmenout A, Tavernier J, Van der Heyden J, Kawashima E, Chollet A, Tizard R, Van Heuverswyn H, Van Vliet A Ruysschaert MR, Fiers W: Molecular cloning of mouse tumor necrosis factor cDNA and its eukaryotic expression. *Nucleic Acids Res* 13:4417–4429, 1985
34. Kaestner KH, Christy RJ, Lane MD: Mouse insulin-responsive glucose transporter gene: characterization of the gene and trans-activation by the CCAAT/enhancer binding protein. *Proc Natl Acad Sci U S A* 87:251–255, 1990
35. Tokunaga K, Taniguchi H, Yoda K, Shimizu M, Sakiyama S: Nucleotide sequence of a full-length cDNA for mouse cytoskeletal β-actin mRNA. *Nucleic Acids Res* 14:2829, 1986
36. Simpson IA, Yver DR, Hissin PJ, Wardzala LJ, Karnieli E, Salans LB, Cushman SW: Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. *Biochim Biophys Acta* 763:393–407, 1983
37. Kelada AS, Macaulay SL, Proietto J: Cyclic AMP acutely stimulates translocation of the major insulin-regulatable glucose transporter GLUT4. *J Biol Chem* 267:7021–7025, 1992
38. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
39. Arbeeny CM, Meyers DS, Hillyer DE, Bergquist KE: Metabolic alterations associated with the antidiabetic effect of β_3-adrenergic receptor agonists in obese mice. *Am J Physiol* 268:E678–E684, 1995
40. Emilsson V, Summers RJ, Hamilton S, Liu YL, Cawthorne MA: The effects of the β_3-adrenoceptor agonist BRL 35135 on UCP isoform mRNA expression. *Biochem Biophys Res Commun* 252:450–454, 1998
41. Yoshitomi H, Yamazaki K, Abe S, Tanaka I: Differential regulation of mouse uncoupling proteins among brown adipose tissue, white adipose tissue, and skeletal muscle in chronic β_3 adrenergic receptor agonist treatment. *Biochem Biophys Res Commun* 253:85–91, 1998
42. Savontaus E, Rouru J, Boss O, Huupponen R, Koulu M: Differential regulation of uncoupling proteins by chronic treatments with β_3-adrenergic agonist BRL 35135 and metformin in obese *fa/fa* Zucker rats. *Biochem Biophys Res Commun* 246:899–904, 1998
43. Stephens JM, Lee J, Pilch PF: Tumor necrosis factor-α-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. *J Biol Chem* 272:971–976, 1997
44. Long SD, Pekala PH: Regulation of GLUT4 mRNA stability by tumor necrosis factor-α: alterations in both protein binding to the 3′ untranslated region and initiation of translation. *Biochem Biophys Res Commun* 220:949–953, 1996
45. Spiegelman BM, Flier JS: Adipogenesis and obesity: rounding out the big picture (Review). *Cell* 87:377–389, 1996