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Chronic effects of AJ-9677 on energy expenditure and energy source utilization in rats

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Abstract

The effects of AJ-9677 on metabolic parameters were examined in rats that had or had not been chronically treated with this β_3-adrenoceptor agonist. A challenge administration of AJ-9677 increased both the temperature of brown adipose tissue and energy expenditure in both groups of rats. However, whereas the former effect was subject to desensitization, the latter effect was augmented by prior chronic administration of AJ-9677. Whereas a challenge administration of AJ-9677 induced a decrease in the respiratory quotient that persisted for at least 15 h in rats pretreated with vehicle, the initial decrease in this parameter lasted for only 4 h in rats pretreated with AJ-9677. These results suggest that, in rats subjected to chronic treatment with AJ-9677, a challenge administration of this drug increased energy expenditure by stimulation not only of fat oxidation but also of glucose oxidation.

Keywords: β_3-adrenoceptor; AJ-9677; Energy expenditure; Respiratory quotient

1. Introduction

The β_3-adrenoceptor, one of three subtypes of mammalian β-adrenergic receptor, is most abundant in adipose tissues (Strosberg, 1997; Robidoux et al., 2004). There are two types of adipose tissue, with the primary function of white adipose tissue being to store excess energy in the form of triglycerides and that of brown adipose tissue being to produce heat. Stimulation of lipolysis in white adipose tissue results in the release of free fatty acids into the circulation and their oxidation in multiple organs and tissues including brown adipose tissue. Both white and brown adipose tissues express the β_3-adrenoceptor, while β_3-adrenergic agonists stimulate lipolysis in white adipose tissue (Liu and Stock, 1995; Germack et al., 1997; Louis et al., 2000) and concomitant heat production in brown adipose tissue (Manara et al., 1996; Malinowska and Schlicker, 1997). Moreover, long-term administration of such agonists results in up-regulation of uncoupling protein in brown adipose tissue (Arbeeny et al., 1995; Emilsson et al., 1998). This protein uncouples fuel oxidation from ATP synthesis (Boss et al., 1998) and thereby increases heat production. β_3-Adrenoceptor agonists are thus potential drugs for the treatment of obesity and obesity-related diseases.

Several β_3-adrenoceptor agonists have been synthesized and their effects on energy homeostasis examined in living animals. Administration of CL 316243 to genetically obese yellow KK mice thus resulted in a reduction in body mass and increased expression of uncoupling protein in white adipose tissue, muscle, and brown adipose tissue (Yoshida et al., 1998). Treatment with AJ-9677 also reduced body mass and ameliorated glucose intolerance in KK-A^y/Ta mice (Kato et al., 2001). Moreover, CL 316243 augmented fat

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oxidation and insulin sensitivity in human subjects (Weyer et al., 1998). Although these findings indicate the clinical potential of β_3-adrenoceptor agonists, the pharmacological properties of such drugs in living animals remain largely uncharacterized.

The respiratory quotient, which is the molar ratio of CO_2 expired to O_2 consumed, reflects the nature of the energy source utilized metabolically in vivo. For oxidation of carbohydrates, the volumes of expired CO_2 and consumed O_2 are equal, yielding a respiratory quotient of 1.0. In contrast, for oxidation of fat, the volume of expired CO_2 is smaller than that of consumed O_2, giving a respiratory quotient of <1.0 (Weir, 1949). Treatment of rats with the β_3-adrenergic agonist BRL 26830A resulted in a decrease in the respiratory quotient, consistent with the notion that such agonists increase energy expenditure by promoting fat oxidation (Wilson et al., 1987).

Drugs do not always exert the same effects when they are administered acutely or chronically, either because of the development of tachyphylaxis or as a result of other adaptations. Differences in the acute and chronic pharmacological effects of AJ-9677 have not been well characterized. We therefore investigated the effects of a bolus administration of AJ-9677 in rats that had or had not been pretreated with this β_3-adrenoceptor agonist for 6 days. We now show that not only fat but also glucose was utilized in response to a bolus administration of AJ-9677 exclusively in rats that had been previously treated with this drug.

2. Materials and methods

2.1. Pharmacological reagents and animals

The β_3-adrenoceptor agonist AJ-9677, or [3-[(2*R*)-[[(2*R*)-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1*H*-indol-7-yloxy]-acetic acid, was synthesized at Dainippon Pharmaceutical. Atenolol and 3-(2-ethylphenoxy)-1-[[(1*S*)-1,2,3,4-tetrahydronaphth-1-yl]amino]-(2*S*)-2-propanol oxalate salt (SR 59230A), antagonists for β_1- and β_3-adrenoceptors, respectively, were obtained from Sigma (St. Louis, MO, USA). For chronic administration of AJ-9677, the drug was suspended in a 0.5% tragacanth solution and administered orally once a day in the morning for 6 days to male Sprague–Dawley rats (body mass, 200 to 300 g) obtained from Clea Japan (Tokyo, Japan). Control animals received only solvent. All animal experiments were performed according to the guidelines of the animal ethics committee of Dainippon Pharmaceutical.

2.2. Assay of intracellular cAMP accumulation

Chinese hamster ovary (CHO) cells stably expressing the rat β_3-adrenoceptor were generated as described previously (Kawashima et al., 1997). The cells were cultured under an atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (ICN Biomedicals, Aurora, OH, USA) supplemented with 10% fetal bovine serum, 100 μM nonessential amino acids (ICN Biomedicals), and G-418 (200 μg/ml) (Life Technologies, Rockville, MD, USA). Cells cultured in 10-cm dishes were detached by washing with phosphate-buffered saline lacking Mg^{2+} and Ca^{2+} followed by incubation for 5 min at 37 °C with phosphate-buffered saline containing 0.5 mM EDTA. The cells were collected by centrifugation at 150×*g* for 5 min at 4 °C and then resuspended in assay buffer containing 1.2 mM $CaCl_2$, 5.3 mM KCl, 0.43 mM KH_2PO_4, 0.48 mM $MgCl_2$, 0.40 mM $MgSO_4$, 130 mM NaCl, 4.1 mM $NaHCO_3$, 0.33 mM Na_2HPO_4, D-glucose (0.98 mg/ml), phenol red (9.8 μg/ml), 3-isobutyl-methylxanthine (43 μg/ml), 0.2% dimethyl sulfoxide, ascorbic acid (170 μg/ml), and 20 mM HEPES–NaOH (pH 7.4). The cell suspension (5×10^4 cells in 100 μl) was mixed with 395 μl of ice-cold assay buffer containing various concentrations of atenolol or SR 59230A and then placed on ice for 10 min, after which 5 μl of assay buffer containing various concentrations of AJ-9677 were added and the mixture was incubated for 30 min at 37 °C. The mixture was then boiled for 5 min and centrifuged at 900×*g* for 5 min at 4 °C, after which the concentration of cAMP in the supernatant was determined with a cAMP enzyme immunoassay system (Amersham Pharmacia Biotech, Little Chalfont, UK).

2.3. Determination of food and water intake and the amount of white adipose tissue

Rats were subjected to chronic administration of AJ-9677 or vehicle for 6 days, and both body mass and the amounts of food and water consumed during the previous 24 h were measured before each administration. After 6 days, epididymal, perinephrial, and retroperitoneal white adipose tissues were removed while the animals are under continuous anesthesia with diethyl ether. The isolated tissue was then weighed.

2.4. Measurement of plasma glucose, free fatty acid, and insulin concentrations in conscious rats

Rats that had been chronically treated with AJ-9677 or vehicle for 6 days were anesthetized with diethyl ether and the abdominal cavity was exposed. A polyethylene cannula (SP31; Natsume, Tokyo, Japan) was inserted into the left iliac artery. The end of the cannula was positioned outside of the body on the back, and the abdominal and dorsal incisions were then sutured. To avoid additional damage, we did not perform venous cannulation for drug administration but rather used the artery cannula for both blood collection and drug injection. Following recovery from surgery for more than 1 h, the animals were injected via the cannula with AJ-9677 (0.1 μg/kg of body mass) dissolved in saline containing 0.01% dimethyl sulfoxide, and blood samples were collected from the cannula at various times thereafter. Plasma concentrations of glucose, free fatty acids, and insulin were measured with the use of a Glucose CII-Test (Wako, Osaka, Japan), a NEFA C-Test (Wako), and an Ultra Sensitive Rat Insulin Kit (Morinaga Institute of Biological Science, Yokohama, Japan), respectively.

2.5. Measurement of temperature around interscapular brown adipose tissue in pithed rats

The following procedures were conducted with rats under continuous anesthesia with diethyl ether. A polyethylene cannula was installed in the trachea, the vagal nerve was severed, and the spinal cord was then destroyed by insertion of a brass rod (2 mm in thickness) into the vertebral column through the left eye, as described previously (Sugimoto et al., 1995). Respiration was

controlled with an artificial respirator (SN-480-7; Shinano Seisakusho, Tokyo, Japan) via the tracheal cannula (4 ml per stroke, 60 strokes/min). The interscapular skin was then cut and a temperature sensor (SXK-67; Technol Seven, Yokohama, Japan) was embedded around the interscapular brown adipose tissue. The cut area was covered with saline-dampened cotton and then with aluminum foil. A temperature sensor (SXK-54; Technol Seven) was also inserted into the rectum. We found that a change in rectal temperature affected heart rate, blood pressure, and the temperature around the brown adipose tissue (data not shown). We therefore positioned the animals under a 100-W lamp and maintained the rectal temperature at 37.0±0.1 °C by adjusting the voltage of the lamp with a power controller. AJ-9677 (0.1 μg/kg) was injected through a cannula that had been inserted into the right saphenous vein. The temperatures of the interscapular brown adipose tissue and the rectum were continuously monitored from immediately before to 70 min after injection. The temperature sensors were connected with a thermistor linearizer (E332-1241-4; Technol Seven) to yield analog signals, which were then converted to digital signals with the Fluclet System version 4.0 (Dainippon Pharmaceutical).

2.6. Determination of respiratory quotient and oxygen consumption in conscious rats

Rats were transferred individually at 0900 h to an animal chamber with a capacity of 6.7 l. The base of the chamber was covered with spruce chips (White Flakes; Charles River Japan, Yokohama, Japan), the chamber was tightly sealed, and air was pumped into the chamber via one of two holes (1000 ml/min). The air flux was monitored with a thermal mass flow meter (model 3750; Kofloc, Kyotanabe, Japan). The chamber was equipped with an electric fan to stir up the air within, which was discharged through the other hole of the chamber into a flow meter, an oxygen sensor (S-3A/DOX; AEI Technologies, Naperville, IL, USA), and a carbon dioxide sensor (CD-2A; AEI Technologies) in series. The analog electrical signals corresponding to the air flux, O_2 concentration and atmospheric pressure, and CO_2 concentration, respectively, were converted to digital signals with the Fluclet System version 4.0.

At 1030 h, the chamber was opened and the rat was orally dosed. The chamber was closed again and the experiment was continued until 0800 h on the next day. Food (CE-2; Clea Japan) and water were available throughout the experiment, and the room temperature was maintained at 26.0 °C. Before and after each experiment, we calibrated the atmospheric pressure and O_2 and CO_2 concentration values. The absolute atmospheric pressure was measured with a precision barometer (Yoshino Keiki, Tokyo, Japan). The absolute value of O_2 concentration was determined from air (20.95% O_2) and from a mixture of N_2 and O_2 containing 18.00% O_2 (Taiyotoyo-Sanso, Ebina, Japan). The absolute value of CO_2 concentration was determined from mixtures of N_2 and CO_2 containing 0.508%, 1.030%, and 2.040% CO_2 (Taiyotoyo-Sanso). Given that air contains 20.95% O_2 and 0.03% CO_2, the respiratory quotient (RQ) was calculated from the equation

$$RQ = (C - 0.0003)/(0.2095 - O)$$

where C and O are the fractional CO_2 and O_2 concentrations. Oxygen consumption (OC, ml min^{-1} $kg^{-0.75}$ at 0 °C and 1 atm) was calculated from the equation

$$OC = PF(0.2095 - O)/M^{0.75}$$

where P is atmospheric pressure (atm), F is air flux (ml/min) corrected to 0 °C, and M is body mass (kg). Energy expenditure (EE, cal min^{-1} $kg^{-0.75}$) (Weir, 1949) was calculated from the equation

$$EE = [3.9 + (1.1 \times RQ)] \times OC.$$

2.7. Measurement of spontaneous motor activity

Rats were transferred individually at 0900 h to a plastic cage [300 mm (width) × 350 mm (length) × 170 mm (height)], the base of which was covered with spruce chips. Each animal was orally dosed at 1030 h, and the experiment was continued until 0800 h on the next day. Food and water were available throughout the experiment, and the room temperature was maintained at 26.0 °C. Spontaneous motor activity of each rat was monitored with the use of an automated apparatus (Supermex; Muromachi Kikai, Tokyo, Japan), which consists of both a sensor mounted above the cage that detects radiant body heat and a computer software system (Masuo et al., 1997). Activity monitored during six 10-min intervals was summed to provide counts per hour.

2.8. Quantitation of uncoupling protein 1 mRNA and histological analysis of epididymal white adipose tissue

Epididymal white adipose tissue was removed from rats anesthetized with diethyl ether. For histological analysis, the tissue was preserved in 10% neutral buffered formalin, and paraffin-embedded sections were subsequently prepared, stained with hematoxylin–eosin, and examined with a light microscope. For determination of the amount of uncoupling protein 1 mRNA, the fresh tissue was stored in RNAlater (Ambion, Austin, TX, USA) and subsequently homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA). Chloroform was added to the homogenate, and total RNA present in the aqueous phase was purified with the use of an RNeasy Mini Kit (Qiagen, Tokyo, Japan). The RNA (0.1 μg in 1 μl) was incubated for 5 min at 65 °C with 1 μl of oligo$(dT)_{12-18}$ primer (0.5 mg/ml, Invitrogen), 1 μl of deoxynucleoside triphosphate mix (10 mM, Invitrogen), and 9 μl of H_2O in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The mixture was then cooled rapidly on ice and subjected to reverse transcription by incubation first at 42 °C for 50 min and then at 70 °C for 15 min with SuperScript II reverse transcriptase (Invitrogen). After treatment with ribonuclease H (Invitrogen), the synthesized cDNA was subjected to the polymerase chain reaction with SYBR Green Master Mix (Applied Biosystems) and specific primers (300 nM). The amplification protocol was performed with an ABI Prism 7700 system (Applied Biosystems) and comprised incubations at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The oligonucleotide primers, synthesized by Sigma Aldrich Japan (Ishikari, Japan), included 5′–ATCTTCTCAGCCGGCGTTT–3′ and 5′–TGGATCTGAAGGCGGACTTT–3′ (forward and reverse, respectively) for uncoupling protein 1 (Bouillaud et al., 1986) and 5′–CCTGTGGCATCCATGAAACTAC–3′ and 5′–ACGGATGTCAACGTCACACTTC–3′ for β-actin (Nudel et al., 1983). The amount of uncoupling protein 1 mRNA was normalized by that of β-actin mRNA.

2.9. Statistical analysis

The median effective concentration (EC_{50}) of AJ-9677 for inducing cAMP accumulation was estimated by nonlinear regression according to the logit model (SAS version 8.2 software; SAS Institute, Cary, NC, USA) for each experiment. Data from multiple experiments were expressed as means ± S.E.M. The area under the curve (AUC) for time course data was calculated and subjected to statistical analysis. The ratio of mRNA was log transformed to improve normality in statistical analysis. Differences between two groups were assessed by Student's two-tailed t test, and those among more than two groups were evaluated by Dunnett's two-tailed multiple-comparison test. The PROBMC function (SAS version 8.2 software) was used to calculate P values for both statistical tests. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of AJ-9677 on intracellular accumulation of cAMP

Treatment of CHO cells that express the rat β_3-adrenoceptor with AJ-9677 resulted in a concentration-dependent increase in the intracellular amount of cAMP (Fig. 1). The maximal response was achieved at 1×10^{-9} M AJ-9677, and the EC_{50} was 1.81×10^{-11} M (95% confidence interval, 1.23×10^{-11} to 2.66×10^{-11} M). Although atenolol (1 μM) had no effect on the concentration–response curve for AJ-9677-induced cAMP accumulation, SR 59230A both shifted the curve to the right and reduced the maximal response in a concentration-dependent manner (Fig. 1). These results are thus consistent with the notion that AJ-9677 is a full agonist for the rat β_3-adrenoceptor.

3.2. Effects of chronic treatment with AJ-9677 on body mass, food and water intake, and body fat in conscious rats

We investigated the effects of daily administration (p.o.) of AJ-9677 (1 mg/kg) for 6 days on body mass, food and water intake,

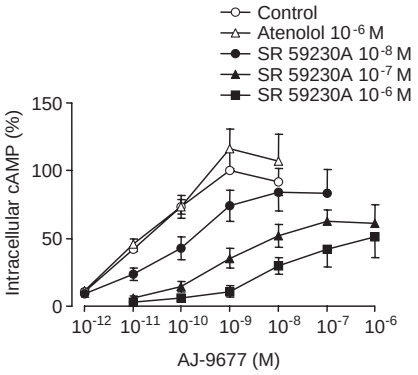

Fig. 1. Effect of AJ-9677 on cAMP production in CHO cells expressing the rat β_3-adrenoceptor. Cells were incubated for 30 min with various concentrations of AJ-9677 in the absence or presence of 0.01 to 1 μM SR 59230A or 1 μM atenolol, as indicated, after which the intracellular concentration of cAMP was determined. Data are expressed relative to the value for cells incubated in the presence of 1 nM AJ-9677 alone and are means ± S.E.M. of values from six independent experiments.

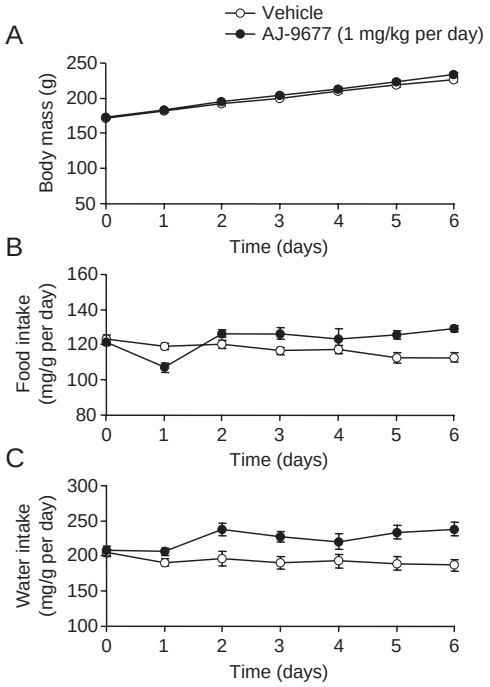

Fig. 2. Effects of AJ-9677 on body mass and on food and water intake in rats. Rats were orally administered either vehicle or AJ-9677 (1 mg/kg) once a day for 6 days, during which time body mass (A), food intake (B), and water intake (C) were determined daily. Food and water intakes were normalized by body mass. Data are means ± S.E.M. of values from 10 rats per group.

and the amount of white adipose tissue in conscious rats. Body mass increased in rats administered AJ-9677 or vehicle and did not differ significantly between the two groups (Fig. 2A). Whereas food intake for AJ-9677-treated rats tended to decrease during the 1st day of drug administration, it returned to normal during the 2nd day and then tended to be higher than that in vehicle-treated animals (Fig. 2B). The water intake of AJ-9677-treated rats tended to increase during the 2nd day of drug administration and to remain increased thereafter (Fig. 2C). The total amount of epididymal, perinephrial, and retroperitoneal white adipose tissue was significantly reduced in rats treated for 6 days with AJ-9677 compared with that in vehicle-treated animals, with the differences in perinephrial and retroperitoneal tissue being most pronounced (Table 1).

3.3. Effects of AJ-9677 on plasma glucose, free fatty acid, and insulin concentrations in conscious rats

We investigated the effects of a bolus intra-arterial injection of AJ-9677 (0.1 μg/kg) on various metabolic parameters in rats that had been subjected to long-term treatment (p.o.) with either AJ-9677 or vehicle. In the vehicle-pretreated animals, the bolus injection of AJ-9677 increased the plasma concentration of glucose by ~120 mg/l within 20 min (Fig. 3A). The plasma glucose concentrations of rats pretreated orally for 6 days with either of two doses of AJ-9677 (0.1 or 1 mg/kg per day) did not differ from those of the vehicle-pretreated animals either before or after the bolus injection of AJ-9677. The AUC for the plot of the increase in plasma glucose concentration (Δglucose) versus time after bolus injection of AJ-9677 also did not differ significantly among the

Table 1
Effects of AJ-9677 on body mass and the amount of white adipose tissue in rats

Treatment	Body mass (g)		Epididymal	Perinephrial	Retroperitoneal	Total WAT (%)
	Initial	Final	WAT (%)	WAT (%)	WAT (%)	
Vehicle	171.8±1.8	225.9±2.5	0.587±0.024	0.091±0.005	0.337±0.025	1.016±0.051
AJ-9677	172.5±1.9	233.1±2.7	0.563±0.032	0.065±0.005	0.168±0.017	0.796±0.049*

Rats were orally administered with either vehicle or AJ-9677 (1 mg/kg) once a day for 6 days, after which the amounts of epididymal, perinephrial, and retroperitoneal white adipose tissue (WAT) were determined and expressed as a percentage of final body mass. Initial and final body mass values are also shown. Data are means±S.E.M. of values from 10 animals per group. *P=0.0059 versus the corresponding value for vehicle-treated animals (Student's t test).

groups pretreated with AJ-9677 or vehicle (Fig. 3B). These results thus showed that a bolus injection of AJ-9677 increased the plasma glucose level and that this effect was not desensitized by chronic drug treatment.

The plasma concentration of free fatty acids in the vehicle-pretreated group was 0.362±0.033 and 0.666±0.037 meq/l before and 10 min after the bolus injection of AJ-9677, respectively, after which the value declined (Fig. 3C). The plasma free fatty acid levels before the bolus injection of AJ-9677 were lower in the AJ-9677-pretreated animals (0.245±0.046 and 0.181±0.022 meq/l in rats pretreated with AJ-9677 at 0.1 or 1 mg/kg per day, respectively) than in the vehicle-pretreated rats. The increase in plasma free fatty acid concentration (ΔFFA) induced by the bolus injection of AJ-9677 was also smaller in the drug-pretreated groups. The AUC of the plot of ΔFFA versus time was thus significantly smaller in the animals pretreated with AJ-9677 (Fig. 3D).

The plasma concentration of insulin in the vehicle-pretreated animals was 2.47±0.21 and 5.28±0.68 ng/ml before and 20 min after the bolus injection of AJ-9677 (Fig. 3E). The plasma insulin concentration before the bolus injection of AJ-9677 was 3.69±0.31 and 1.73±0.23 ng/ml in rats pretreated with AJ-9677 at 0.1 or 1 mg/kg per day, respectively. The insulin response to the bolus injection of AJ-9677 was attenuated in the AJ-9677-pretreated animals. The AUC for the time course of the increase in plasma insulin concentration (Δinsulin) was thus significantly reduced by pretreatment of rats with AJ-9677 (Fig. 3F). These results showed that a bolus injection of AJ-9677 increased both free fatty acid and insulin levels in plasma, and that chronic treatment with AJ-9677 induced desensitization to these effects.

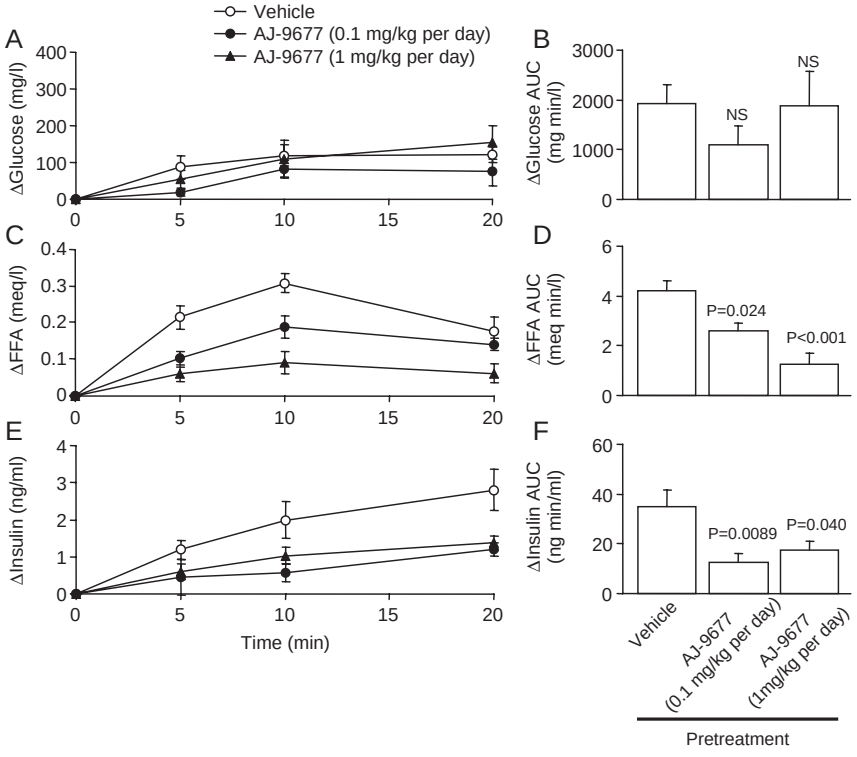

Fig. 3. Effects of AJ-9677 on plasma concentrations of glucose, free fatty acids, and insulin in conscious rats. Rats pretreated (p.o.) once a day for 6 days with either vehicle or AJ-9677 at doses of 0.1 or 1 mg/kg per day were injected intra-arterially on day 7 with AJ-9677 (0.1 μg/kg), and the plasma concentrations of glucose, free fatty acids, and insulin were monitored at various times thereafter. The time courses of the increases (Δ) in the plasma concentrations of glucose, free fatty acids (FFAs), and insulin are shown in (A), (C), and (E), respectively, and the corresponding AUCs are shown in (B), (D), and (F). Data are means±S.E.M. of values from six animals per group. P values for the indicated comparisons with the vehicle-pretreated group were determined by Dunnett's test. NS: not significant.

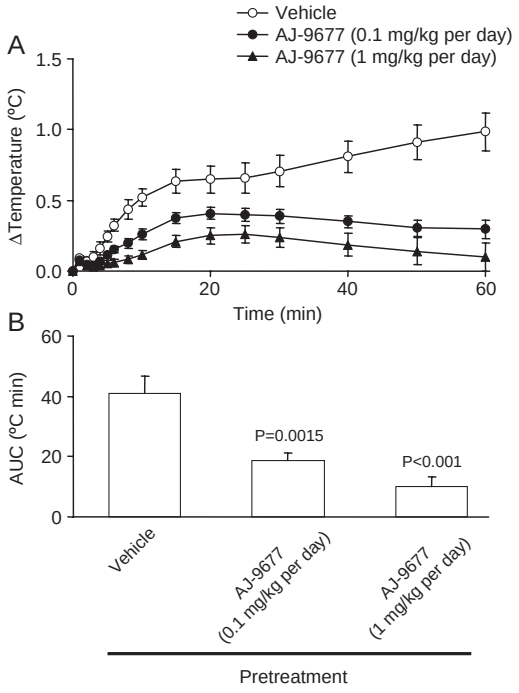

Fig. 4. Effect of AJ-9677 on heat production by brown adipose tissue in pithed rats. Rats pretreated (p.o.) once a day for 6 days with either vehicle or AJ-9677 at doses of 0.1 or 1 mg/kg/day were injected intravenously on day 7 with AJ-9677 (0.1 μg/kg), and the temperature around interscapular brown adipose tissue was monitored at various times thereafter. The time courses of the increase (Δ) in brown adipose tissue temperature are shown in (A) and the corresponding AUCs are shown in (B). Data are means ± S.E.M. of values from eight animals per group. *P* values for the indicated comparisons with the vehicle-pretreated group were determined by Dunnett's test.

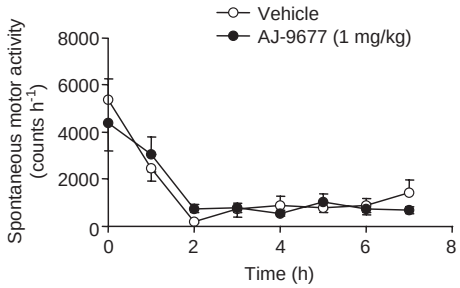

Fig. 6. Lack of effect of AJ-9677 on spontaneous motor activity in naive conscious rats. Rats were orally administered either vehicle or AJ-9677 (1 mg/kg), after which the time course of spontaneous motor activity was monitored. Data are means ± S.E.M. of values from eight rats per group.

3.4. Effect of AJ-9677 on brown adipose tissue temperature in pithed rats

We next investigated the effect of a bolus intravenous injection of AJ-9677 on heat generation in rats that had been chronically treated with vehicle or AJ-9677. The temperature around interscapular brown adipose tissue before the bolus injection of AJ-9677 (0.1 μg/kg) was 35.05 ± 0.23 °C, 35.65 ± 0.23 °C, and 35.43 ± 0.19 °C in rats pretreated with vehicle or AJ-9677 at 0.1 or 1 mg/kg per day, respectively; these values did not differ significantly. The bolus injection of AJ-9677 increased the temperature around interscapular brown adipose tissue in vehicle-pretreated rats by 0.71 ± 0.11 °C at 30 min and by 0.98 ± 0.13 °C at 60 min, at which time the effect had still not plateaued (Fig. 4A). In contrast, the bolus injection of AJ-9677 induced maximal temperature increases of only 0.41 ± 0.04 °C at 20 min or 0.26 ± 0.06 °C at

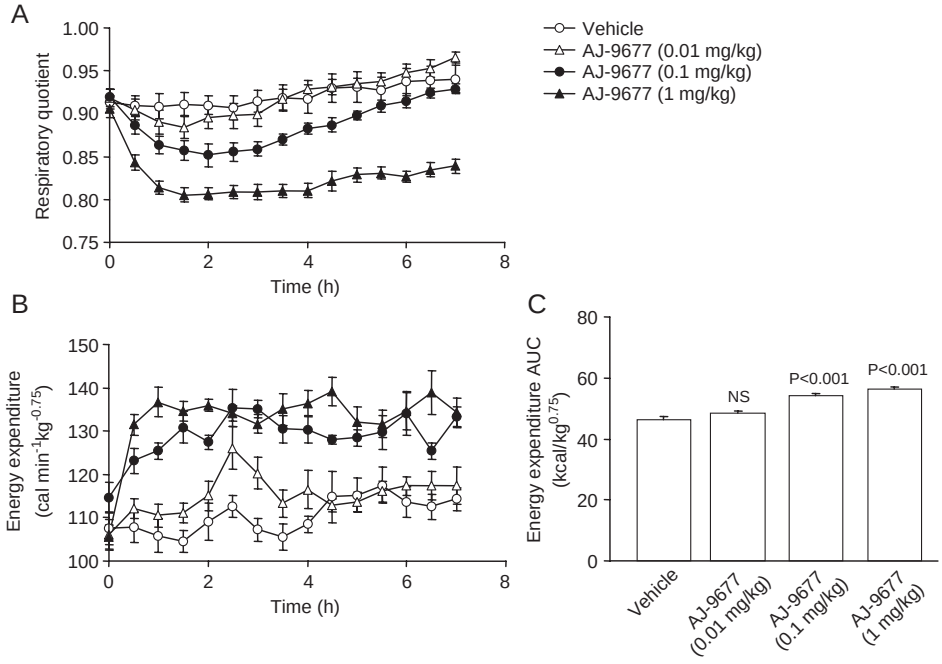

Fig. 5. Effects of AJ-9677 on the respiratory quotient and energy expenditure in naive conscious rats. Rats were orally administered either vehicle or AJ-9677 at doses of 0.01, 0.1, or 1 mg/kg, after which the time courses of the respiratory quotient (A) and energy expenditure (B) were monitored. The corresponding AUCs for energy expenditure were also determined (C). Data are means ± S.E.M. of values from eight rats per group. *P* values for the indicated comparisons with the vehicle-treated group were determined by Dunnett's test.

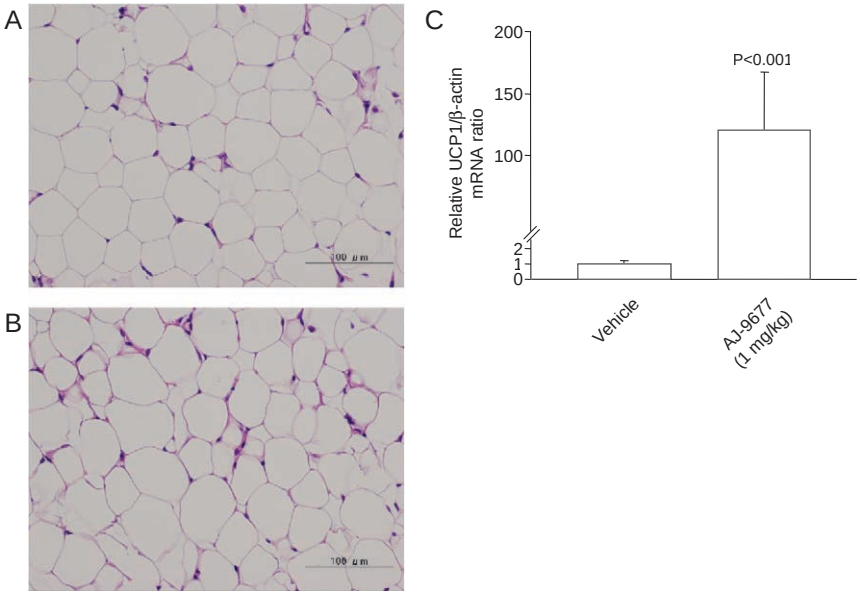

Fig. 7. Effects of AJ-9677 on the histology of, and the abundance of uncoupling protein 1 mRNA in, epididymal white adipose tissue in naive rats. Rats were orally administered either vehicle or AJ-9677 (1 mg/kg) and epididymal white adipose tissue was removed 5 h later. The tissue from vehicle-treated (A) or AJ-9677-treated (B) animals was stained with hematoxylin–eosin and examined by light microscopy. The amount of uncoupling protein 1 (UCP1) mRNA in the tissue was also determined, normalized by that of β-actin mRNA, and expressed relative to the normalized value for vehicle-treated rats (C). Data in (C) are means±S.E.M. of values from six animals per group. The *P* value for comparison with the vehicle-treated group was determined by Student's *t* test.

25 min in rats pretreated with AJ-9677 at 0.1 or 1 mg/kg per day, respectively. The AUC for the time course of the increase in brown adipose tissue temperature was thus significantly reduced by pretreatment of rats with AJ-9677 (Fig. 4B), suggesting that chronic treatment with AJ-9677 resulted in desensitization to the effect of the drug on heat production.

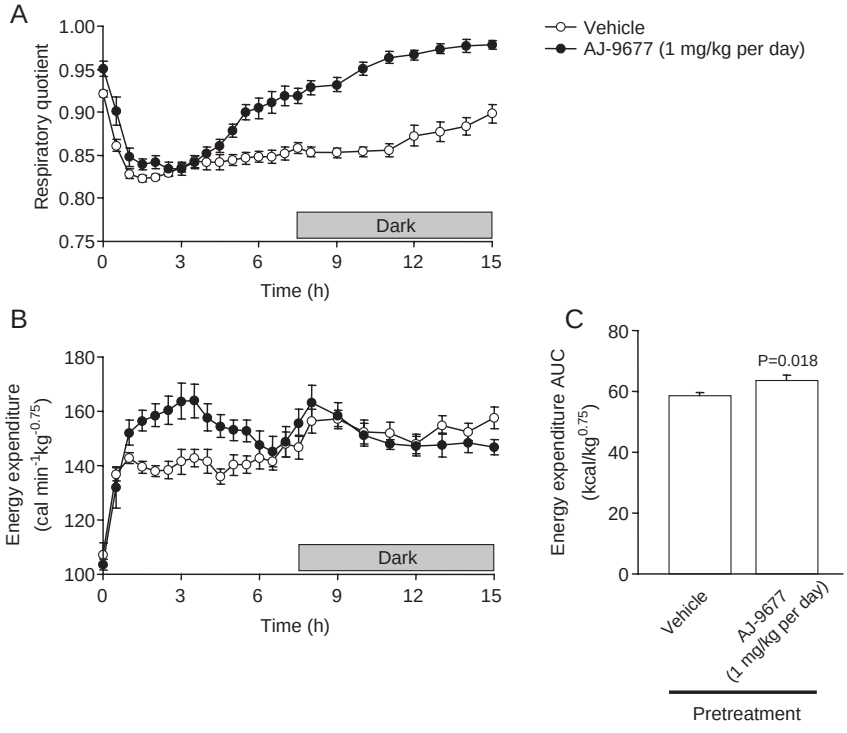

Fig. 8. Effects of AJ-9677 on the respiratory quotient and energy expenditure in AJ-9677-pretreated conscious rats. Rats were orally administered either vehicle or AJ-9677 (1 mg/kg) once a day for 6 days. On day 7, all animals received AJ-9677 (1 mg/kg, p.o.) and the time courses of the respiratory quotient (A) and energy expenditure (B) were monitored. The animals were maintained in the dark after 7.5 h, as indicated. The corresponding AUCs for energy expenditure between 0 and 7 h after drug challenge were also determined. Data are means±S.E.M. of values from eight rats per group. The indicated *P* value for comparison with the vehicle-pretreated group was calculated by Student's *t* test.

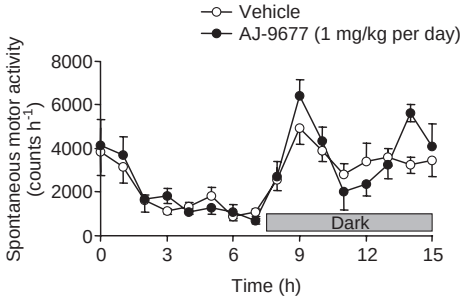

Fig. 9. Effect of AJ-9677 on spontaneous motor activity in AJ-9677-pretreated conscious rats. Rats were orally administered either vehicle or AJ-9677 (1 mg/kg) once a day for 6 days. On day 7, all animals received AJ-9677 (1 mg/kg, p.o.) and the time course of spontaneous motor activity was monitored. The animals were maintained in the dark after 7.5 h, as indicated. Data are means ± S.E.M. of values from eight rats per group.

3.5. Effects of AJ-9677 on the respiratory quotient and energy expenditure in conscious rats

We next investigated the effects of acute oral administration of AJ-9677 on the respiratory quotient and energy expenditure in conscious rats. The respiratory quotient of rats that received vehicle was maintained between 0.91 and 0.94 (Fig. 5A). Whereas a bolus administration of AJ-9677 at 0.01 mg/kg did not markedly affect the respiratory quotient, that of AJ-9677 at 0.1 or 1 mg/kg resulted in a substantial decrease in this parameter. The energy expenditure of rats that received vehicle was maintained between 104 and 118 cal min^{-1} $kg^{-0.75}$ (Fig. 5B). Administration of AJ-9677 at 0.01 mg/kg did not affect energy expenditure, whereas that at 0.1 or 1 mg/kg resulted in a marked increase in this parameter. The AUC for the plot of energy expenditure versus time was significantly greater for rats treated with AJ-9677 at 0.1 or 1 mg/kg than for those that received vehicle (Fig. 5C). These data thus showed that a bolus administration of AJ-9677 reduced the respiratory quotient and increased energy expenditure in a dose-dependent manner in naive rats.

Acute administration of AJ-9677 (1 mg/kg) did not affect the spontaneous motor activity of rats (Fig. 6), indicating that the effect of this drug on energy expenditure was not attributable to an increase in ambulatory activity. Bolus administration of AJ-9677 also had no effect on the histology of epididymal white adipose tissue apparent 5 h after treatment (Fig. 7A, B), but it did induce a significant increase in the amount of uncoupling protein 1 mRNA in this tissue (Fig. 7C).

We next compared the effects of bolus administration of AJ-9677 on the respiratory quotient and energy expenditure in rats that had been chronically treated with AJ-9677 or vehicle. The challenge administration of AJ-9677 at 1 mg/kg reduced the respiratory quotient in both vehicle- and AJ-9677-pretreated animals (Fig. 8A). However, whereas the decrease in the respiratory quotient persisted for at least 15 h in rats chronically treated with vehicle, it had begun to reverse by 4 h and was no longer apparent at 10 h in the AJ-9677-pretreated group. These results thus suggested that the energy source for the rats pretreated with AJ-9677 was predominantly glucose by 10 h after the challenge administration of AJ-9677.

The challenge administration of AJ-9677 increased energy expenditure in rats pretreated with either vehicle or AJ-9677 (Fig. 8B). This effect persisted for at least 15 h in both groups of animals, although the values for the AJ-9677-pretreated rats were greater than those for the vehicle-pretreated rats between 1 and 6 h after the drug challenge. The AUC for the time course of energy expenditure between 0 and 7 h after the challenge injection was significantly greater for rats pretreated with AJ-9677 than for those pretreated with vehicle (Fig. 8C).

There was no difference in spontaneous motor activity after the challenge administration of AJ-9677 between the vehicle- and

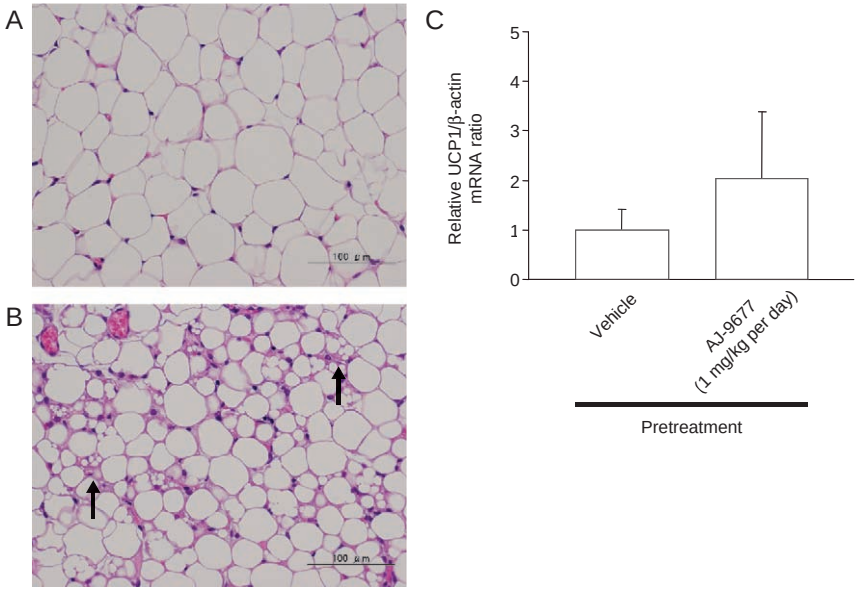

Fig. 10. Effects of AJ-9677 on epididymal white adipose tissue in AJ-9677-pretreated conscious rats. Rats were orally administered either vehicle or AJ-9677 (1 mg/kg) once a day for 6 days. On day 7, all animals received AJ-9677 (1 mg/kg, p.o.) and epididymal white adipose tissue was removed 5 h later. The tissue from vehicle-pretreated (A) and AJ-9677-pretreated (B) animals was stained with hematoxylin–eosin and examined with a light microscope. Arrows indicate multilocular cells. The amount of uncoupling protein 1 (UCP1) mRNA in the tissue was also determined, normalized by that of β-actin mRNA, and expressed relative to the normalized value for vehicle-pretreated rats (C). Data in (C) are means ± S.E.M. of values from six animals per group.

drug-pretreated groups (Fig. 9), showing that ambulatory activity was not responsible for the difference in energy expenditure. Histological analysis revealed that the size of adipocytes was reduced and that the cells manifested a multilocular morphology in the epididymal white adipose tissue isolated from rats chronically treated and challenged with AJ-9677 compared with animals pretreated with vehicle (Fig. 10A, B). The chronic administration of AJ-9677 thus appeared to induce brown adipocyte-like features in white adipose tissue, a phenomenon that has previously been observed in rats treated with other β_3-adrenoceptor agonists (Himms-Hagen et al., 1994; Yoshida et al., 1998). In contrast, the abundance of uncoupling protein 1 mRNA did not differ after AJ-9677 challenge between the vehicle- and drug-pretreated groups (Fig. 10C).

4. Discussion

The acute and chronic effects of β_3-adrenergic stimulation on the abundance of β_3-adrenoceptor mRNA have been found to differ (Granneman and Lahners, 1992; Revelli et al., 1992; Arbeeny et al., 1995; Bengtsson et al., 1996). It is therefore important to investigate both short- and long-term pharmacological effects of β_3-adrenergic agonists in order to characterize fully the responses to these drugs and their clinical potential. We have now investigated the effects of a bolus administration of AJ-9677 on metabolic parameters in rats that had or had not been chronically exposed to this β_3-adrenoceptor agonist. The challenge injection of AJ-9677 induced similar increases in the plasma concentration of glucose within 20 min in both rats pretreated with AJ-9677 and those pretreated with vehicle. In contrast, Grujic et al. (1997) previously showed that the β_3-adrenergic agonist CL 316243 reduced blood glucose levels in mice. Similar to the effect of AJ-9677 in the present study, CL 316243 increased the plasma concentration of insulin (Grujic et al., 1997). The increase in plasma insulin concentration in the previous study was much greater than that observed in the present study, however, possibly accounting, at least in part, for the difference in the effects on glucose levels between the two studies.

The mechanism by which β_3-adrenergic agonists stimulate insulin secretion remains unclear. Evidence suggests that free fatty acids in plasma increase the cytosolic Ca^{2+} concentration in pancreatic β cells by activating GPR40, a G protein-coupled receptor expressed in these cells, and thereby stimulate insulin secretion (Itoh et al., 2003; Kotarsky et al., 2003). The increase in the circulating levels of free fatty acids induced by β_3-adrenergic agonists might thus contribute to the effect of these drugs on insulin secretion. Indeed, the CL 316243-induced increase in the serum concentration of free fatty acids observed by Grujic et al. (1997) was much larger than the increase in the plasma concentration of free fatty acids induced by AJ-9677 in the present study. The mechanism responsible for the AJ-9677-induced increase in blood glucose levels also remains unknown. β-Adrenergic agonists stimulate glycogenolysis and gluconeogenesis in the liver and thereby increase glucose levels (Hers and Hue, 1983; Pilkis et al., 1988). However, the β_3-adrenoceptor is not expressed in the liver (Strosberg, 1997). Given that glycerol, a product of lipolysis, serves as a substrate for gluconeogenesis in the liver, the increased production of glycerol induced by AJ-9677 might contribute to the associated increase in the plasma glucose concentration.

The increases both in the plasma free fatty acid level and in the temperature of brown adipose tissue induced by the challenge injection of AJ-9677 were smaller in rats pretreated with this drug than in those pretreated with vehicle, probably as a result of desensitization to the effects of β_3-adrenergic agonists on lipolysis and accumulation of cAMP in white adipose tissue (Atgié et al., 1998; Vicario et al., 1998) and brown adipose tissue (Jockers et al., 1998). However, the increase in energy expenditure induced by AJ-9677 was not subject to desensitization, and was actually potentiated by prior treatment with the drug. It is possible that released free fatty acids generated by lipolysis are taken up and oxidized by adipocytes or skeletal muscle more rapidly in rats chronically treated with AJ-9677. However, we cannot exclude the possibility that the AJ-9677-induced increase in energy expenditure in rats chronically treated with this drug is largely independent of lipolysis and subsequent release of free fatty acids in white adipose tissue and of β-oxidation in brown adipose tissue. Clarification of this issue will require evaluation of the release and uptake of free fatty acids.

A challenge administration of the β_3-adrenoceptor agonist FR-149175 was recently shown to increase oxygen consumption to a greater extent in Zucker fatty rats after chronic treatment with this drug (Hatakeyama et al., 2004). However, it is not possible to determine fuel source by measurement of oxygen consumption alone. We therefore measured expired CO_2 in addition to consumed O_2 and thereby determined the time courses of both the respiratory quotient and energy expenditure. We thus found that, although AJ-9677 challenge induced similar rapid decreases in the respiratory quotient in both drug- and vehicle-pretreated rats, the respiratory quotient of AJ-9677-pretreated animals rebounded much faster than did that of those pretreated with vehicle. The respiratory quotient of the AJ-9677-pretreated group thus reached 0.95 within 10 h after drug challenge, indicating that the energy source at this time point was almost exclusively glucose. This difference between the two groups of animals did not appear attributable simply to desensitization to the pharmacological effects of AJ-9677, given that the increase in energy expenditure was maintained for at least 15 h in both groups. Thus, soon after the challenge administration of AJ-9677, both groups of animals appeared to utilize fat as an energy source, but the energy source of the AJ-9677-

pretreated rats was shifted back to glucose much sooner than was that of the control animals. Stimulation of β-adrenoceptors in brown adipose tissue increases the oxidation not only of fat but also of glucose (Isler et al., 1987; Wilson et al., 1987). The mechanism by which chronic treatment with AJ-9677 affects this timing of the shift in energy source from fat back to glucose remains to be determined.

In summary, we have shown that various effects of a challenge administration of AJ-9677 differed between rats that had or had not been chronically treated with this drug. In particular, the challenge administration of AJ-9677 increased energy expenditure by inducing the burning not only of fat but also of glucose in rats subjected to long-term pretreatment with this drug. Elucidation of the underlying mechanism of this phenomenon may provide insight into the regulation of energy homeostasis and therefore into the pathogenesis of obesity and obesity-related disorders.

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