Acute and chronic effects of FR-149175, a $\beta_3$-adrenergic receptor agonist, on energy expenditure in Zucker fatty rats

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$\beta_3$-ADRENERGIC RECEPTOR ($\beta_3$-AR) agonists have great potential as antiobesity drugs, since they stimulate lipolysis in white adipose tissues (fuel supply) and concomitantly increase whole body energy expenditure by enhancing energy dissipation through uncoupling protein (UCP) 1 stimulation (1, 2, 11, 14, 22). $\beta_3$-AR agonists are also effective in treating diabetes, presumably by a normalization of the secretion of adipocytokines from adipocytes that influence insulin sensitivity like adiponectin and TNF-$\alpha$ (17).

In clinical therapy of obese and obese diabetic patients, maintenance of reduced body weight is required after the successful reduction of body weight resulting from calorie control, exercise, or medication. However, biological responses mediated by $\beta$-ARs are associated with functional desensitization (16, 30). Increased phosphorylation of $\beta$-ARs is believed to play an important role in agonist-promoted uncoupling, which leads to rapid desensitization and later to down-regulation of the receptor. For $\beta$-ARs, this phenomenon has been investigated intensely in $\beta_2$-ARs. Unlike $\beta_1$-ARs and $\beta_2$-ARs, earlier evidence indicates that $\beta_3$-ARs are relatively resistant to desensitization and downregulation, probably resulting from the lack of most of the serine/threonine residues phosphorylated by $\beta$-AR kinase and by protein kinase A in a classical process of desensitization (3, 12, 21, 25, 28). In line with these reports, Collins et al. (6) reported that the beneficial effects of $\beta_3$-AR agonists in decreasing body weight and fasting plasma glucose levels can persist even after many weeks of chronic treatment. Aitgi et al. (2), however, showed adrenergic desensitization of lipolytic response after chronic administration of a $\beta_3$-AR agonist. Although stimulation of whole body energy expenditure and lipid mobilization are considered to be important for the antiobesity effect of $\beta_3$-AR agonists, few studies have been performed to elucidate whether the stimulatory effect of $\beta_3$-AR agonists on energy expenditure is negatively influenced by chronic exposure to agonists. We therefore examined acute and chronic effects of a $\beta_3$-AR agonist on whole body oxygen consumption in genetically obese Zucker fatty rats. We previously reported that FR-149175, ethyl $\{(S)-8-[(R)-2-(3-chlorophenyl)-2-hydroxy-ethyl]-6,7,8,9-tetrahydro-5H-benzo[c]lohepton-2-yl]-acetate$ monohydrochloride monohydrate, a selective $\beta_3$-AR agonist, has antiobesity and antidiabetic effects (8, 32). It activates recombinant human $\beta_3$-ARs expressed in CHO cells with a higher potency against $\beta_3$-AR than against $\beta_1$-AR and $\beta_2$-AR by factors of 380 and >630 times, respectively. We used this selective $\beta_3$-AR agonist to clarify the influence of acute and chronic exposure to agonists on whole body oxygen consumption.

**MATERIALS AND METHODS**

**Animals and Diet**

Male Zucker fa/fa rats (10–12 wk old) were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). Animals were housed in stainless steel hanging rack cages. The animal room was maintained on a 12:12-h light-dark cycle (0700–1900) at a temperature of 21–25°C. Food (CRF-1; Oriental Yeast, Tokyo, Japan) and water were provided ad libitum. All animal experimental procedures were performed according to the guidelines of the Animal Experiment Committee of Fujisawa Pharmaceutical.

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Experimental Design

Study 1: Lipolytic effect of FR-149175 in Zucker fatty rats. Rats were divided into four groups (3 rats/group) based on body weight. FR-149175 was administered orally (0.32, 1, and 3.2 mg/kg). Blood samples were taken from the tail vein with heparinized capillaries (Bayer Medical, Tokyo, Japan) at 0, 1, 3, and 6 h after drug treatment. Plasma was separated by centrifugation (12,000 rpm, 10 min, 4°C) and free fatty acid (FFA) in plasma was measured using the Wako NEFA C-Test (Wako Pure Chemical Industries, Osaka, Japan).

Study 2: Antiobezity effect of FR-149175 in Zucker fatty rats. Zucker fa/fa rats were divided into five groups (8 rats/group) based on body weight. FR-149175 was administered orally (0.1, 0.32, 1, and 3.2 mg/kg) two times daily (0900 and 1700) for 2 wk. Body weight and food intake were measured every day. After a 2-wk treatment, blood was collected as described in the lipolysis study. Retroperitoneal white adipose tissue (RTWAT) was dissected and weighed.

Study 3: Acute and chronic effects of FR-149175 on oxygen consumption. Rats were divided into four groups (4 rats/group) based on body weight. FR-149175 was administered orally (0.1, 1, and 3.2 mg/kg), and oxygen consumption was measured every 30 min over 3 h. The following day, rats were dosed two times a day for 2 wk, and oxygen consumption was measured every week. After completion of measurement of oxygen consumption in the 2nd week, rats were killed, and RTWAT was dissected for measurement of mRNA levels.

Measurement of Oxygen Consumption

Whole body oxygen consumption was assessed in up to four rats simultaneously using an Oxymax (Columbus Instruments, Columbus, OH). Rats were placed in the chamber and kept for 3 h for stabilization. Flow rates were 2 l/min with a 45-s sampling time at 15-min intervals. Oxygen consumption values for the last 1 h of the stabilization period were used for calculation of the basal value expressed as the average of four values obtained from measurement every 15 min. After dosing drugs or vehicle, measurement was performed for a subsequent 3 h. Oxygen consumption values measured every 30 min were used in calculations. Food and water were not available during measurement. Results were obtained as mass-adjusted consumption (ml·kg⁻¹·h⁻¹) and expressed as percent changes against basal values.

Assessment of β3-ARs, UCP1, hormone-sensitive lipase, carnitine palmitoyltransferase-1, and long-chain acyl-CoA dehydrogenase mRNA levels in RTWAT

mRNA levels were determined by RT-PCR. Total RNA was extracted using an RNeasy Mini Kit (QIAGEN, Tokyo, Japan). To remove potential contaminating genomic DNA, extracted RNA was treated with RNase-free DNase I (Takara Bio, Shiga, Japan) according to the manufacturer’s protocol. cDNA was synthesized from 1 µg purified RNA through RT reaction where RT reagent mixture consisted of AmpliTag Gold, and isolated RNA was incubated at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min for 1 cycle (GeneAmp PCR System 2400; Perkin-Elmer, Tokyo, Japan). cDNA was added to PCR reagent mixture, SYBR Green Master Mix, with the upstream and downstream primers (200 nM each). PCR was performed at 95°C for 15 s and 60°C for 1 min for 40 cycles on an ABI PRISM 7700 (Applied Biosystems Japan, Tokyo, Japan). RT-PCR for 18S rRNA was included as control. Oligonucleotide primers used (synthesized and supplied by SIGMA Genosys Japan, Hokkaido, Japan) were β3-ARs (forward primer 5'-GCCGAGACTACAGACCAT-3', reverse primer 5'-TGCATGGCCGTTCTCATG-3'), UCP1 (forward primer 5'-ATCTTCTCAGCCGGGTTT-3', reverse primer 5'-TGGATCTGACCCGGGACTTT-3'), hormone-sensitive lipase (HSL: forward primer 5'-AAGTGTGAGCCGCGCTATCCAG-3', reverse primer 5'-ATCACGCCCAATGGCCTT-3'), carnitine palmitoyltransferase (CPT)-1 (forward primer 5'-AGTTCACTCCGGTTCAAGATGG-3', reverse primer 5'-ATCACCCACCGACGATA-3'), long-chain acyl-CoA dehydrogenase (LCAD; forward primer 5'-GGAAAGCCGGAGAAGGTGAGTAGA-3', reverse primer 5'-GCCATGTTCTCTGCAATTGATG-3'), and 18S rRNA (forward primer 5'-TGCATGGCCGTTCTCATG-3', reverse primer 5'-TAGCATGGCAAGAGTCGTT-3'). mRNA levels were expressed in arbitrary units (AU), taking values in vehicle-treated rats as 100 AU.

Drug

FR-149175 was synthesized in the Medicinal Chemistry Research Laboratories at Fujisawa Pharmaceutical. It was dissolved in water and given orally in a volume of 5 ml/kg. AmpliTaq Gold and ×10 PCR Gold Buffer with dNTP and SYBR Green PCR Master Mix were obtained from Applied Biosystems Japan.

Data and Statistical Analysis

Values are presented as means ± SE. Statistical analysis was carried out by one-way ANOVA, and statistical significance of the differences among groups was determined by Dunnett’s multiple-comparison test. Values of P < 0.05 were regarded as significant.

RESULTS

Study 1: Lipolytic Effect of FR-149175 in Zucker Fatty Rats

FR-149175 at a dose of 0.32 mg/kg had little or no effect on plasma FFA levels, but definite increases were observed at 1 mg/kg or higher followed by return to the basal level by 6 h (Fig. 1). Taking this result into consideration, we decided to give 0.1, 0.32, 1, and 3.2 mg/kg FR-149175, two times daily, in a subsequent repetitive-administration study.

Study 2: Antiobezity Effect of FR-149175 in Zucker Fatty Rats

FR-149175 dose dependently inhibited body weight gain, and significant inhibition was observed at 3.2 mg/kg two times daily in the later part of the experimental period (Fig. 2A).

Fig. 1. Lipolytic effect of FR-149175. Drug was administered orally to fasted Zucker fatty rats, and blood samples were taken at the indicated time points. Data are means ± SE of 3 rats/group. FFA, free fatty acid. **Significantly different from control at P < 0.01.
Higher doses (1 and 3.2 mg/kg 2 times daily) reduced food intake, which recovered to the initial level within 4 days (Fig. 2B). RTWAT weight was slightly reduced at 1 mg/kg, and a significant decrease was detected at 3.2 mg/kg (Fig. 2C).

Study 3: Acute and Chronic Effects of FR-149175 on Oxygen Consumption

Oxygen consumption. Results in Fig. 3 show that, after drug administration, whole body oxygen consumption increased rapidly in a dose-dependent manner, and it remained elevated until 3 h at 1 mg/kg or higher. From the following day, daily drug treatment was started to evaluate the influence of chronic treatment on oxygen consumption. Oxygen consumption was measured weekly for 2 wk. The second and third measurements indicated that the thermogenic response was not attenuated by chronic exposure of rats to the agonist. Furthermore, in contrast to attenuation, the thermogenic response was enhanced drastically over time in rats administered 3.2 mg/kg two times daily.

β3-ARs, UCP1, HSL, CPT-1, and LCAD Gene Expression in RTWAT

β3-AR mRNA levels were measured to determine whether chronic treatment caused up- or downregulation of β3-ARs. As indicated in Fig. 4A, chronic treatment with FR-149175 at any dose had little or no effect on β3-AR mRNA levels. We also measured several transcripts in RTWAT thought to be involved in energy expenditure and fuel supply to elucidate the mechanisms of augmentation of thermogenic response provided by the higher dose of FR-149175. As shown in Fig. 4B, UCP1 mRNA levels were increased markedly in rats treated with FR-149175, especially at the highest dose. There was a 52.5-fold increase at 1 mg/kg and tremendous upregulation at 3.2 mg/kg (640-fold increase). Additionally, chronic treatment dose dependently increased HSL mRNA levels (115.8, 151, and 197.4% compared with vehicle-treated rats at 0.1, 1, and 3.2 mg/kg, respectively). In contrast, for transcripts implicated in β-oxidation, neither CPT-1 nor LCAD mRNA levels were changed from repetitive administration of FR-149175 (Fig. 4, D and E).

DISCUSSION

Exposure of Zucker fatty rats to FR-149175, a β3-selective AR agonist, for 2 wk did not cause functional desensitization of the thermogenic response. The highest dose of FR-149175 induced a progressive increase in thermogenic response accompanied by upregulation of UCP1 and HSL gene expression in the visceral fat depot.

First, the lipolytic effect was evaluated to determine doses for the study examining the antiobesity effect of repetitive administration of FR-149175. In the study evaluating the antiobesity effect, a decrease in food intake was observed for a few days after starting administration at doses of >1 mg/kg, corresponding to doses that induce a definite lipolytic effect. This is in agreement with several earlier reports concerning another selective β3-AR agonist (2, 18, 23). FR-149175 dose dependently and significantly reduced body weight gain and decreased visceral fat pad weight. From these results, it is likely that the transient decrease in food intake contributed to reduced body weight gain. Also, it is apparent that repetitive administration at doses that induce lipolysis by single administration is able to produce an antiobesity effect. The present results are also in good agreement with our previous report where the drug was given one time a day (32).
Single administration of FR-149175 produced a dose-related increase in whole body oxygen consumption in Zucker fatty rats. This energetic response was not attenuated during the 2-wk treatment period. Prolonged exposure of β3-ARs to adrenergic agonists is reported to lead to the downregulation of receptors, which accompanies the reduction in mRNA and protein levels (16, 30). However, the present study demonstrated that there was no attenuation of the thermogenic response at any dose and no decrease in β3-AR mRNA levels in RTWAT. These findings agree with earlier studies reporting that β3-ARs, unlike β1- and β2-ARs, are relatively resistant to desensitization and downregulation (3, 12, 21, 25, 28).

Quite interestingly, and the opposite to what might be predicted, rats treated with the highest dose developed thermogenic capability gradually. Earlier reports demonstrated that treatment of rats with β3-AR agonists resulted in increased UCP1 gene expression in brown adipose tissue, the major organ responsible for nonshivering thermogenesis in rodents. However, several recent studies demonstrated that the mechanisms for the antiobesity effect by β3-AR agonists may involve the induction of brown adipocytes in white adipose tissue in addition to stimulating thermogenesis of original brown fat depots, resulting in an increase in the thermogenic capacity (4, 5, 7, 9, 10, 24, 27). Ectopic UCP1 gene expression is a more likely mechanism for the drastic enhancement of thermogenic response, and therefore a few transcripts in white fat pads that are known to participate in the fuel supply and energy expenditure were measured. UCP1 mRNA levels in RTWAT of vehicle-treated rats were very low, whereas in rats treated with FR-149175, ectopic expression of UCP1 was detected. In particular, the highest dose of FR-149175, which exhibited a definite reduction in body weight and retroperitoneal fat pad weight, induced a drastic increase in UCP1 gene expression. These findings agree with accumulating evidence that β3-AR agonists can induce brown adipocytes in white fat depots, which leads to an increase in thermogenic capability of the animal. Collins et al. (6) and Guerra et al. (15) suggested that the ability of mice to reduce obesity with β3-AR agonists is correlated with the capability of induction of brown adipocytes in the white fat pad. For the molecules involved in fuel supply, HSL gene expression was dose dependently upregulated. Therefore, it can be postulated that the ectopic expression of UCP1 in combination with an increase in HSL gene expression in white fat pads greatly contributes to the enhancement of energy expenditure and body weight reduction.

Another possibility that accounts for the gradual increase in thermogenic responses other than induction of brown adipocytes in white adipose tissue is activation of relatively dormant brown adipocytes in a population of white adipocytes. In patients with pheochromocytoma whose circulating norepinephrine concentrations are high, UCP gene transcription and brown adipocytes in abdominal fat are reactivated (20, 26). Similar responses could have occurred in rats with repetitive administration of FR-149175. In humans, brown adipose tissue disappears as they grow, and its mass is little compared with rodents. However, the fact that β3-AR agonists increase expression of UCP1 in white fat depots suggests that they might be effective in increasing energy expenditure, even in humans by inducing brown adipocytes in white adipose tissue and/or activating relatively dormant brown adipocytes in white adi-

Fig. 3. Acute and chronic effects of FR-149175 on oxygen consumption in Zucker fatty rats. After estimation of basal values, drug was administered orally, and oxygen consumption was subsequently measured. Drug treatment was continued and measurement repeated weekly for 2 wk. A: 1st day; B: 8th day; C: 15th day of treatment. Values are means ± SE of 4 rats/group.
pose tissue. This is supported by evidence that β-AR agonists are able to increase UCP mRNA levels in adipocytes of adult humans (4).

Recent work by Granneman et al. (13) has demonstrated the contribution of UCP1-independent thermogenesis, resulting from mitochondrial biogenesis and induction of genes involved in lipid oxidation, to the thermogenic effect of a β-AR agonist. We evaluated some transcripts involved in mitochondrial β-oxidation. CPT-1 catalyzes esterification of long-chain acyl-CoAs to L-carnitine for transport into mitochondria, a critical step of mitochondrial β-oxidation. Pharmacological stimulation of CPT-1 activity leads to increased energy production, indicating that β-oxidation could play an important role in whole body energy expenditure as well as energy dissipation through UCPs (29). LCAD is reported to play a key role in β-oxidation of unsaturated fatty acids (19). In the present study, transcriptional upregulation was not detected for either CPT-1 or LCAD, suggesting that the β-AR stimulation is a regulatory factor for UCP1 but not for genes involved in mitochondrial β-oxidation in white adipose tissue. This finding agrees with an earlier report demonstrating that leptin but not norepinephrine upregulates CPT-1 mRNA (31) but disagrees with another report demonstrating chronic treatment of UCP1 knockout mice with CL-316243 increases LCAD gene expression (13). Further studies are required to determine whether β-oxidation is transcriptionally activated by β-AR agonists.

The present results, however, do not exclude the implication of enhanced mitochondrial β-oxidation in a progressive increase in energy expenditure. One intriguing point revealed in the present study is that HSL gene expression was upregulated by chronic treatment. It thus seems likely that chronic exposure to β-AR agonists could result in increased lipolysis capacity resulting from transcriptional upregulation of HSL genes, in addition to activation of HSL through cAMP. Enhancement of lipolytic activity results in the activation of mitochondrial β-oxidation, even if genes implicated in mitochondrial β-oxi-

Fig. 4. Chronic effect of FR-149175 on gene expressions in RTWAT of Zucker fatty rats. RNA was isolated and evaluated for expression of β-ARs (A), uncoupling protein 1 (UCP1; B), hormone-sensitive lipase (HSL; C), carnitine palmitoyltransferase-1 (CPT-1; D), and long-chain acyl-CoA dehydrogenase (LCAD; E) by quantitative RT-PCR. Data are means ± SE of 4 rats/group. Significantly different from control at *P < 0.05 and **P < 0.01.
dation are not upregulated, and increased UCP1 also helps the β-oxidation system produce heat.

It seems unlikely that potentiation of thermogenesis by chronic treatment is the result of accumulation of drugs in blood, since no accumulation was observed in Sprague-Dawley rats dosed 56 mg·kg⁻¹·day⁻¹ for 13 wk (data not shown).

In summary, the present study demonstrates that acute treatment of obese Zucker rats with FR-149175 produces both lipolysis and a dose-related increase in oxygen consumption in the same dose range. After chronic treatment, body weight and lipolysis and a dose-related increase in oxygen consumption in /H9252-oxidation system produce heat. 

\[ \text{dose} \times \text{antiobesity effect} \]

\[ \text{UCP1 gene expression in white adipose tissue in combination with the enhanced gene expression of HSL.} \]

**REFERENCES**


